ACTA PHYSIOLOGICA SCANDINAVICA

ACTA PHYSIOL. SCAND.

Vol. 69. INDEX

Fasc 1—2 (January—February 1967)

Fasc 3 (March 1967)

Fasc 4 (April 1967)

Vol. 69. INDEX

Fasc. 1—2. (January—February 1967)

Fasc 3 (March 1967)

INDEX AUCTORUM

Acta physiol. scand. 1967. 69. 1—12

From the Department of Histology, Karolinska Institutet, Stockholm, Sweden

Extraneuronal Binding of Catecholamines and 3,4-Dihydroxyphenylalanine (dopa) in Salivary Glands

By

Bertil Hamberger, Karl-Axel Norberg and Lars Olson

Received 28 February 1966

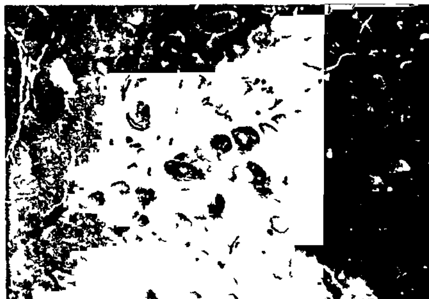
Abstract

Hamberger, B., K.-A. Norberg and L. Olson. *Extraneuronal binding of catecholamines and 3,4-dihydroxyphenylalanine (dopa) in salivary glands.* Acta physiol. scand. 1967. 69. 1—12.

importance related to transmitter mechanisms

Several tissues have the ability to take up and bind CA[1], and it is now generally accepted that this occurs predominantly in the adrenergic nerves. Denervation of the salivary glands, however, does not completely abolish the binding of NA (Andén, Carlsson and Waldeck 1963, Fischer, Kopin and Axelrod 1965) and tyramine (Carlsson and Waldeck 1963, Fischer *et al.* 1964, Almgren, Andén and Waldeck 1965) which suggests that the amines are bound partly to extraneuronal sites. After reserpine pretreatment, the uptake by a postganglionically sympathectomized salivary gland may be even larger than by an innervated gland, provided that MAO and COMT have been inhibited (Andén *et al.* 1963). In view of these data is was considered of interest to localize the sites of the extraneuronally bound CA. Studies were therefore performed on the binding of CA and dopa in salivary glands *in vivo* and *in vitro*, using the histochemical method of Falck and Hillarp (Falck *et al.* 1962, Falck 1962, Corrodi and Hillarp 1963, 1964).

[1] Abbreviations used: CA = catecholamines, A = adrenaline, NA = noradrenaline, DA = dopamine, dopa = 3,4-dihydroxyphenylalanine, MAO = monoamine oxidase, COMT = catechol-O-methyl transferase.



F g 1 Submax llary (top) and subl ngual (bottom) gland rat L dopa 100 mg/kg 1 h No or very eak spec fic fluorescence nduced by L dopa can be seen in the parenchyma To the left a blood vessel w th intensely fluorescent nerve term nals 130 ×



Fig 2 Submax llary (t p) subl ngual botto n gland rat n alam de 500 mg kg 4 h H 22/54 500 mg/kg 1 1/2 h and pu l K g kg 1 h An intense fluorescence is found in both the acini and ducts of the two glands. N e he swollen appearance of the submax llary gland in wh ch the terminals are almost maske l t e flu res ence in the parenchyma 130



Fig 3

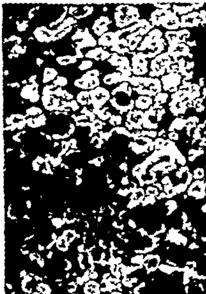


Fig 4

Fig 3 Sublingual gland rat nialamide 500 mg/kg, 4 h, H 22/54 500 mg/kg 1 1/2 h, L-dopa 100 mg/kg, 1 h The non innervated acini exhibit a strong fluorescence while a few large ducts show a less intense fluorescence Some small blood vessels with surrounding nerve terminals can also be seen 130 ×

Fig 4 Sublingual gland, rat untreated *in vivo* Slices of the gland were incubated with L-dopa 10 µg/ml A strong fluorescence was induced by L-dopa in some acini and in a few acini only the peripheral part shows fluorescence The lack of uptake in certain areas is probably due to experimental conditions of the *in vitro* system 130 ×

Material and Methods

(Ilidar®, Hoffmann La Roche) L-dopa, D-dopa (—) and (—) NA hydrochloride — α methyl-NA hydrochloride (Corbasil®, Hoechst Anilin AB) DA hydrochloride α methyl DA hydrochloride (Hässle) and (—) A bitartrate The doses of CA were calculated as free base All *in vivo* drugs were administered intraperitoneally except L-dopa, which was given subcutaneously

In vivo experiments For number of animals and treatments see Table I The animals were killed

least two experiments for each type of treatment and each experiment included 2 rats Control rats

immediately in cold Krebs Ringer bicarbonate medium containing glucose and saturated in an



Fig 5

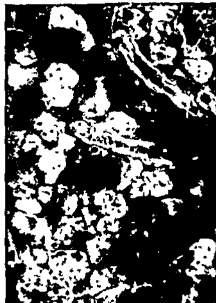


Fig 6

Fig 5 Submaxillary gland rat nialamide 500 mg/kg 4 h, H 17/27 250 mg/kg 2 h, DA 50 mg/ 1 h 130 ×

Fig 6 Submaxillary gland rat nialamide 500 mg/kg 4 h H 22/54 500 mg/kg 1 1/2 h, DA 50 mg/ 1 h 210

In the acini of figs 5 and 6 there is a strong fluorescence while in the striated ducts the spec fluorescence if any is considerably less intense

[illegible]

slices were then removed from the incubation bath, immediately frozen, freeze-dried in small tis dryers with a high capacity (Thieme 1965) and prepared for fluorescence microscopy as with *in vivo* preparations All experiments were performed with control slices from the same animal T tissue pieces from every treatment were always taken for fluorescence microscopy At least two se rate experiments were generally performed for each type of treatment

Results

In vivo (Table I No or only a slight increase in the normal fluorescence in the saliva glands *cf* Norberg and Olson 1965) was found after treatment with L-dopa alo (Fig 1), or if the animals had been treated with MAO- or COMT-inhibitors alo before the L-dopa The administration of L-dopa in combination with both MA and COMT inhibitors resulted in a strong fluorescence in the parenchyma (Fig and 3)

Green to yellow green fluorescence in glands from DA-injected animals could found when the animals had been pretreated with a combination of MAO and COM inhibitors (Fig 5 and 6 The fluorescence was very intense in the acini, but le intense in the ducts Essentially the same appearance was found after treatment wi a COMT-inhibitor in combination with *α*-methyl-NA DA caused no or only a fai

TABLE I Extraneuronal fluorescence in salivary glands induced by drugs *in vivo* (Number of animals within brackets)

Drugs[1]		Fluorescence intensity relative to controls in the same experiments Submaxillary and sublingual glands: Acini	Ducts
L dopa	(6)	no or slight increase	no or slight increase
Nialamide + L dopa	(6)	no or slight increase	no or slight increase
H 22/54 (2) or H 17/27 (4) + L dopa	(6)	no or slight increase	no or slight increase
Nialamide + H 22/54 (2) or H 17/27 (4) + L dopa	(6)	marked increase[1]	marked increase[1]
DA	(6)	no increase	no increase
Nialamide + DA	(6)	no increase	no increase
H 17/27 + DA	(4)	no increase	no increase
Nialamide + H22/54 (2) or H 17/27 (4) + DA	(6)	marked increase[1]	slight increase[1,2]
α methyl NA	(4)	no increase	no increase
H 17/27 + α methyl NA	(4)	marked increase	slight increase

Drugs doses and time before killing
nialamide 500 mg/kg 4 hrs H 22/54 500 mg/kg 1 1/2 hr H 17/27 250 mg/kg 2 hrs L-dopa 100 mg/kg 1 hr DA 50 mg/kg 1 hr α methyl NA 25 mg/kg 1 [illegible] hr The animals receiving α methyl NA were given the α receptor blocking agent azapetine phosphate 15 mg/kg 30 min before *α methyl NA*

[1] No increase in ligated glands (2)

[2] Small strongly fluorescent cells were found in the excretory ducts of the submaxillary gland

fluorescence when one of the inhibitors of MAO or COMT was given alone nor did any fluorescence occur after DA or α methyl NA alone

In vitro (Table II) The present studies showed that in reserpine pretreated animals incubation with L dopa NA or α methyl NA in concentrations from 1 to 10 μg/ml,

TABLE II Extraneuronal fluorescence in salivary glands induced by drugs *in vitro*

(Number of experiments within brackets)

Treatment in vivo	Treatment in vitro			Fluorescence intensity relative to controls in the same experiment	
				Submaxillary gland	
Drugs[1]	Drugs	μg/ml		Acini	Ducts
Reserpine[2]	L-dopa	0.1	(2)	No increase	No increase
		1	(2)	No increase	Slight increase
		10	(2)	Slight increase[4]	Marked increase
Reserpine[2] + Nialamide	L-dopa	0.1	(3)	No increase[3,4]	No increase[3,4]
		1	(7)	No increase[3,4]	Slight increase[3,4]
		10	(5)	Slight increase[3,4]	Marked increase[3,4]
Reserpine + Nialamide	D-dopa	1	(3)	No increase	No increase
		10	(3)	No increase	No increase
Reserpine	α methyl-DA	1	(4)	No or slight increase	No increase
		10	(4)	Marked increase[4]	Slight increase
Reserpine[2]	NA	0.1	(4)	No increase	No increase
		1	(4)	No increase	Slight increase
		10	(4)	Slight increase	Marked increase
Reserpine[2] + Nialamide	NA	0.1	(4)	No increase	No increase
		1	(5)	No or slight increase	Slight increase[4]
		10	(5)	Slight increase[4]	Marked increase[4]
Reserpine + Nialamide + H 22/54 or H 17/27	NA	0.1	(3)	No increase	No increase
		1	(3)	No or slight increase	Slight increase
		10	(3)	Slight increase[4]	Marked increase
Reserpine + Nialamide	NA	0.1	(1)	No increase	No increase
		1	(2)	No increase	Slight increase
		10	(1)	Slight increase	Marked increase
Reserpine[2]	α methyl-NA	0.03–0.1	(6)	No increase[4]	No increase[4]
		1–3	(15)	No or slight increase[4]	Slight increase[3,4]
		10	(11)	Slight increase[3,4]	Marked increase[3,4]
Reserpine	2,4-dinitrophenol 10^-4 M no glucose + α methyl-NA	1	(4)	No increase	Slight increase
		10	(4)	No increase	Marked increase

TABLE II Cont

Treatment in vivo Drugs[1]	Treatment in vitro Drugs	μg/ml		Fluorescence intensity relative to controls in the same experiment: Submaxillary gland, Acini	Ducts
Reserpine	Desmethylimipramine 10⁻⁴M + α methyl NA	1	(4)	No increase	Slight increase
		10	(4)	No increase	Marked increase
Reserpine	Desmethylimipramine 10⁻⁴M + α methyl NA	1—10	(4)	No increase	No increase
Reserpine + Nialamide	A	1	(2)	No increase	Slight increase[6]

[1] Drugs doses and time before killing
reserpine 5—10 mg/kg 12—18 hrs nialamide 100 mg/kg 2—4 hrs
H 22/54 500 mg/kg 2 hrs H 17/27 250 mg/kg 1½ hr
[2] Same result without reserpine pretreatment (2)
[3] No increase in ligated glands (2)
[4] Same result after preincubation with NSD 1015 50 μg/ml (3)
[5] Marked increase in acini of the sublingual gland (2)
[6] Small strongly fluorescent cells were found in the excretory ducts of the submaxillary gland

caused an increased green to yellow-green fluorescence in the parenchyma of the salivary glands In the submaxillary gland the fluorescence was most intense in the striated ducts, and a slight fluorescence was found in the acini (Fig 7 and 10 control Fig 12), especially after 10 μg/ml After incubation with α methyl DA on the other hand a more intense fluorescence was found in the acini than in the striated ducts (Fig 11) In the sublingual gland the most intense fluorescence was generally found in the acini (Fig 4) No extraneuronal fluorescence was observed after incubation with the same drugs in concentrations of 0 1 μg ml No difference was found in the extraneuronal binding after L dopa NA or α methyl NA if pretreatment with reserpine was excluded The fluorescence after L dopa or NA was not increased by pretreatment with a MAO inhibitor Preincubation with the decarboxylase inhibitor NSD 1015 failed to diminish binding after L-dopa indicating that L dopa was not converted to any great extent to DA before binding D-dopa caused no fluorescence, while (+)-NA apparently behaved similarly to (−) NA The extraneuronal fluorescence intensity after NA was not further increased if a COMT inhibitor was given in addition to a MAO inhibitor Dinitrophenol (10⁻⁴M) combined with exclusion of glucose from the medium or desmethylimipramine (10⁻⁴M) prevented



Fig 7



Fig. 8



Fig 9

Fig 7—9 Submaxillary gland, rat, reserpine 10 mg/kg, 12 hrs Slices of the gland were incubated with α methyl-NA, 10 μg/ml, in Fig 7 without preincubation and in Fig 8 and 9 after preincubation with desmethylimipramine in concentrations of 10 [illegible]M and 10 [illegible]M respectively
In Fig 7 a strong fluorescence due to α-methyl NA is seen in the nerve terminals (↗) and ducts, while the acini exhibit fluorescence of moderate intensity In Fig 8 no specific fluorescence is seen in nerve terminals and acini, while some is seen in the striated ducts In Fig 9 no specific fluorescence can be seen 130 ×

extraneuronal fluorescence from occurring in acini after α-methyl-NA (Fig. 8), while the fluorescence in the striated ducts was unchanged No extraneuronal fluorescence was found not even in the ducts after preincubation with desmethylimipramine in a higher concentration (10 [illegible]M) (Fig 9).

In salivary glands where the main excretory ducts had been ligated and the acini allowed to atrophy (Junquiera 1951, Andén, Norberg and Olson 1966), no extraneuronal fluorescence was found after the *in vivo* injection of L-dopa or DA, or *in vitro* incubation with L-dopa or α-methyl-NA (Fig 13)

Both *in vivo* and *in vitro*, small strongly fluorescent cells were sometimes found in the epithelium of the large excretory ducts (Fig 14) These cells were found alike after DA, NA, α-methyl-NA and A, but never in untreated controls

Discussion

The specificity of the histochemical fluorescence reaction used has been well established in several papers Falck *et al* 1962, Corrodi and Hillarp 1963, 1964, Norberg and Hamberger 1964) The fluorescence found in the parenchyma of the salivary glands after the injection of CA or L dopa is thus derived from corresponding dihydroisoquinolines L-dopa, which causes a strong fluorescence in the parenchyma, may have been converted wholly or partly to DA or NA during the experiment This could well have been the case *in vivo* but such a conversion could hardly have occurred to



Fig 10

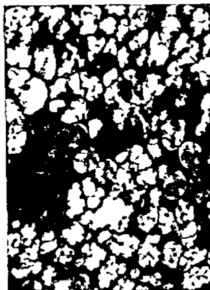


Fig 11

any great extent *in vitro* since no difference was found if the tissue had been preincubated with the dopa decarboxylase inhibitor NSD 1015 Under these conditions a marked decarboxylase inhibition is likely to occur This is supported by the observation, that preincubation with NSD 1015 in the concentration used in the present experiments prevents fluorescence from appearing in the pericytes of cerebral capillaries in brain slices incubated with L dopa (Hamberger 1967, to be publ)

Certain other differences between the *in vivo* and the *in vitro* results were also found Binding after the injection of L dopa, for instance was found *in vivo* to similar extents in the acini and striated ducts of the submaxillary gland while the *in vitro* binding was most prominent in the ducts Also *in vivo* both MAO and COMT had to be inhibited in advance in order to obtain a marked binding after the injection of L dopa or DA Accordingly, no binding was found with α methyl NA *in vivo* unless a COMT-inhibitor was given in advance *In vitro* enzyme inhibition was not necessary A possible explanation of these discrepancies is that *in vivo* CA or L dopa could be metabolized outside the salivary glands, *e g* in the liver and this may lower their concentration at the site of binding during the experiment Also, the doses given *in vivo* are difficult to compare with the *in vitro* concentrations Other explanations may be related to the time intervals for administration of the drugs, and that under *in vitro* conditions secretory activity is not modulated by nerve impulses Dinitrophenol and the inhibitor of neuronal uptake desmethylimipramine (10 [illegible] M) inhibited the bind-

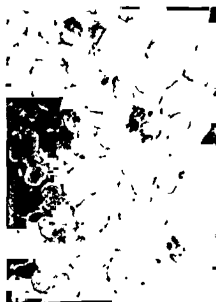


Fig. 12



Fig. 13

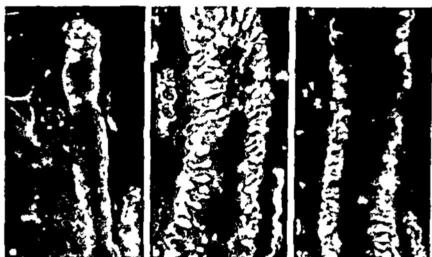


Fig. 14. Submaxillary gland, rat, reserpine 10 mg/kg 12 h, nialamide 100 mg/kg 2 h. Slices of the gland were incubated with NA 1 μg/ml (14 a) or A 1 μg/ml (14 b, c). Small cells in the large ducts of the gland exhibit a strong fluorescence. 210

ing of α-methyl-NA to acini but not to ducts. It thus seems likely that differences in the mechanism of binding may be responsible for some of the differences observed between the acini and ducts. The finding that MAO inhibition did not appreciably

increase extraneuronal binding of NA *in vitro* was somewhat surprising, since MAO is known to occur in the parenchyma of the glands (Almgren *et al* 1966) Since other experiments show that MAO is active intraneuronally under the present *in vitro* conditions (Hamberger 1967, to be publ), it seems possible that the present results can be attributed to a considerably lower extraneuronal MAO activity, which is insufficient to metabolize the NA in the parenchymal cells — Irrespective, however, of these discrepancies between the results obtained *in vivo* and *in vitro*, the present histochemical data, obtained with two experimental procedures, show that an extra neuronal reserpine resistent binding of catecholamines and in all probability also of L dopa can be obtained in salivary glands

The *in vivo* results on the binding of DA and NA are in good agreement with the results of Andén *et al* (1963) who showed that both MAO and COMT had to be inhibited in order to obtain any considerable extraneuronal binding of labelled NA Only a small extraneuronal binding can be obtained in animals which were not pretreated in this way (Anden *et al* 1963, Fischer *et al* 1965) The present doses of CA are considerably higher than those used in studies with labelled compounds where extraneuronal binding has been found (Anden *et al* 1963, Fischer *et al* 1964) The use of such high doses proved necessary if a visible fluorescence in the parenchyma was to be found It is probable however, that the present results demonstrate the same extraneuronal binding sites as have been found by biochemical techniques Thus Fischer *et al* (1964) found that tyramine was taken up extraneuronally *in* salivary glands in amounts which appeared to be proportional to the dose given within a wide dose range up to levels comparable with those used in the present study

The extraneuronal binding in salivary glands may be of importance as a source of error in the evaluation of biochemical measurements but its possible relation to physiological conditions is somewhat obscure Ligation of the excretory ducts which causes a severe atrophy of the acini (Junqueira 1951 Almgren *et al* 1966) and decreases secretory and metabolic activity prevents extraneuronal binding after injection or incubation with CA or L-dopa This suggests that the state of activity in the parenchyma possibly secretory activity is of importance for the binding However there are also other possibilities In the brain capillaries small cells pericytes and or endothelial cells have been found to accumulate DA after the administration of L dopa (Bertler Falck and Rosengren 1963) and to take up CA in brain slices Hamberger and Masuoka 1965) These cells may have an important function for the inactivation of CA in the brain It is possible that the salivary gland parenchyma has a similar function CA being metabolized in the parenchymal cells after physiological release from the adrenergic nerve terminals Furthermore Almgren Anden and Waldeck (1965) have suggested from their data that extraneuronal binding may be important also for neuronal uptake since they found a reduced uptake of tyramine in ligated glands where the adrenergic nerves showed no apparent changes (Anden *et al* 1966) It is thus possible that extraneuronal binding is of importance for adrenergic transmitter mechanisms

The investigation has been supported by a research grant (B67 12x 714 02) from the Swedish Medical Research Council

[illegible]

[illegible] Research Ltd England ([illegible])

References

Almgren O, N E Andén and B Waldeck Extraneuronal binding as a possible factor of tyramine uptake by sympathetic nerves *Life Sci* 1965 *4* 121—126

Almgren O, N E Andén J Jonason K A Norberg and L Olson Cellular localization of monoamine oxidase in rat salivary glands *Acta physiol scand* 1966 *67* 21—26

Andén N E A Carlsson and B Waldeck Reserpine resistent uptake mechanisms of noradrenaline in tissues *Life Sci* 1963 *2* 889—894

Andén N E K A Norberg and L Olson The adrenergic nerves of rat salivary glands after excretory duct ligation *Acta physiol scand* 1966 *66* 501—506

Bertler A B Falck and E Rosengren The direct demonstration of a barrier mechanism in the brain capillaries *Acta pharmacol* (Kbh) 1963 *20* 317—321

Carlsson A and B Waldeck β hydroxylation of tyramine in vivo *Acta pharmacol* (Kbh) 1963 *20* 371—374

[illegible]

Falck B, Observations on the possibilities of the cellular localization of monoamines by a fluorescence method *Acta physiol scand* 1962 *56* Suppl 197

Falck B N Å Hillarp G Thieme and A Torp Fluorescence of catecholamines and related compounds condensed with formaldehyde *J Histochem Cytochem* 1962 *10* 348—354

[illegible]

[illegible] *J Pharmacol exp Ther* 1963 *141* 161—165

Fischer J J Musacchio I Kopin and J Axelrod Effects of denervation on the uptake and β hydroxylation of tyramine in the rat salivary gland *Life Sci* 1964 *3* 413—419

Hamberger B T Malmfors and Ch Sachs The standardization of paraformaldehyde and of certain procedures for the histochemical demonstration of catecholamines *J Histochem Cytochem* 1965 *13* 147

Hamberger B and D Masuoka Localization of catecholamine uptake in rat brain slices *Acta pharmacol* (Kbh) 1965 *22* 363—368

Junqueira L C Cytological cytochemical and biochemical observations on secreting and resting salivary glands *Exp Cell Res* 1951 *2* 327 338

Norberg K A and B Hamberger The sympathetic adrenergic neuron Some characteristics revealed by histochemical studies on the intraneuronal distribution of the transmitter *Acta physiol scand* 1964 *63* Suppl 238

Norberg K A and L Olson Adrenergic innervation of the salivary glands of the rat *Z Zellforsch* 1965 *68* 183 189

Thieme G Small tissue dryers with high capacity for rapid freeze-drying *J Histochem Cytochem* 1965 *13* 386 389

Acta physiol. scand 1967. 69. 13—22

From the Laboratory of Chemical Pharmacology, National Heart Institute, National Institutes of Health, Bethesda 14, Maryland, USA

The Effect of Immunosympathectomy on the Retention and Metabolism of Noradrenaline

By

FOLKE SJOQVIST[1], PALMER W TAYLOR, Jr,[2] and ELWOOD TITUS

Received 7 June 1966

Abstract

SJOQVIST, F, P TAYLOR and E TITUS *The effect of immunosympathectomy on the retention and metabolism of noradrenaline* Acta physiol scand 1967 *69* 13—22

The retention of radioactive noradrenaline (NA) was measured in different organs of immunosympathectomized (I S) mice 30 min after i v injection of dl NA 7 ^{3}H (17 9 or 1 79 μg/kg S A 4 7 C/mmole) A striking decrease in the capacity to retain NA ^{3}H was observed in the heart (1 6 per cent of controls) and the submaxillary gland (4 1 per cent of controls) Immunosympathectomy was only partially effective in the intestine and colon (33 and 47 per cent of normal retention

and vas deferens Metabolites, for the most part methylated accounted for a larger precentage of the total ^{3}H in the extracts from tissues of I S animals compared to controls In the I S submaxillary glands of both the mouse and the rat the radioactivity retained after injection occurred

normal organ

Recent studies have shown that the growth of mammalian sympathetic neurons depends upon a specific protein, which can be isolated from several tissues and reaches particularly high concentrations in mouse salivary glands (Levi Montalcini and Cohen 1956, 1960, Cohen 1960, Levi-Montalcini 1964) If newborn animals are injected with an antiserum to the specific nerve growth factor an extensive destruction of sympathetic ganglia occurs with concomitant degeneration of the peripheral sympathetic innervation (Levi-Montalcini and Booker 1960)

Chemical changes following immunosympathectomy (I S) include a decrease in noradrenaline (NA) levels and monoamine oxidase activity in spleen, lung heart

[1] Present address Department of Pharmacology Karolinska Institutet Stockholm 60 Sweden
[2] To whom correspondence about this paper should be addressed

and submaxillary glands, but not in brain (Levi-Montalcini and Angeletti 1962, Klingman 1965). Examination of formaldehyde treated tissues from immunosympathectomized rats under the fluorescence microcope according to Falck *et al* (1962) has shown virtually complete absence of neuronal NA in peripheral effector organs innervated by the superior cervical and paravertebral ganglia By contrast, fluorescent nerve terminals were demonstrated in vas deferens and, to a lesser extent, in the intestine (Hamberger *et al.* 1964, 1965) The same investigators found a[illegible] increased number of fluorescent tissue cells in the intestine of immunosympathec[illegible] tomized rats compared to controls The characteristics of the increased fluorescenc[illegible] suggested that it was due to 5-hydroxytryptamine.

Since immunosympathectomy promises to be a useful pharmacological tool, i[illegible] seemed of interest to assess its effectiveness by additional means The ability t[illegible] concentrate NA from the circulation into tissue stores that are protected fro[illegible] metabolism is characteristic of noradrenergic tissues (Hertting and Axelrod 1961 Hertting *et al* 1961, Potter *et al* 1965) This function has therefore been tested in th[illegible] organs of immunosympathectomized and normal animals by measuring the re[illegible] tention of radioactive NA 30 min after the injection of d, l-NA-7-^{3}H The chemica[illegible] and chromatographic studies described here indicate that the ability to take up N[illegible] is almost entirely lost in heart and submaxillary gland, reduced in spleen, colo[illegible] and intestine and increased in vas deferens The retention of O-methylated metab[illegible] olites of NA was increased in vas deferens and submaxillary gland but not ap[illegible] preciably changed in other tissues by immunosympathectomy

A preliminary account of part of this work has appeared (Sjöqvist *et al* 1965) an[illegible] similar results have been independently reported from other laboratories (Iverse[illegible] *et al* 1965, Zaimis *et al* 1965)

Methods

Sympathetic nerve growth factor was extracted from mouse submaxillary glands purified and used for immunization of rabbits as previously described by Cohen (1960) and Levi Montalcin[illegible]

[illegible]

least one subject of each experimental litter These revealed that the antiserum had produced [illegible] approximately 85 [illegible] destruction of the

[illegible]

nerve endings In animals treated with antiserum the dense cored vesicles characteristic of a[illegible]

[illegible] vas deferens but only

[illegible]

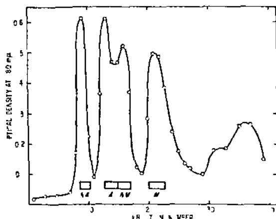


Fig 1 Chromatography on Whatman P 20 Cellulose phosphate paper of a mixture containing 0 5 μmol each of noradrenaline (NA), adrenaline (A) normetanephrine (NM) and metanephrine (M) according to the procedure of Roberts (1962) Cross hatched bars indicate length of paper strip occupied by component as indicated by inspection under ultraviolet lamp O, Absorption of 280 mμ of individual 0 9 cm transverse section of paper

the background

In order to determine NA ³H levels the remaining supernatant solutions were made up to a volume of 20 ml with 0 4 N perchloric acid and NA was separated from the O methylated metabolites by

Losses of the metabolites by these procedures were negligible After lyophilization of the aqueous phase the residue was taken into 0 5 ml of water transferred to a small conical tube and concentra

amounts of ultraviolet absorbing impurities near the lower end (see for example Fig 1) The papers were then washed with alcohol and deionized water allowed to dry in air and stored for later use After chromatography the carrier compounds were located by examination of the air dried paper under a low wavelength ultraviolet lamp and the strips were then cut into transverse sections 0 9 cm wide These were placed in a thin quartz windowed holder designed to fit the Beckman spectrophotometer and the ultraviolet absorbtion of each section at 280 mμ was compared

TABLE I Retention of total radioactivity and NA—³H in tissues of normal and immunosympathectomized mice 30 min after i.v. injection of (±)—NA—7—³H (17.9 μg/kg). Each value represents an average from 4 animals ± SEM

	μ μmoles, of labeled compound per g of tissue			
	Normal		Immunosympathectomized	
Organ	Total	NA—³H	Total	NA—³H
Vas deferens	44.8 ± 2.4	26.7 ± 9.9	92.9 ± 12	49.1 ± 11.3
Colon	77.9 ± 9.2	52.5 ± 4.8	62.9 ± 3.5	24.7 ± 2.1
Intestine	93.5 ± 5.5	30.1 ± 3.4	77.3 ± 4.6	9.9 ± 0.3
Spleen	37.3 ± 4.8	20.0 ± 1.7	34.6 ± 3.0	15.1 ± 2.0
Heart	376 ± 18	342 ± 31	24.6 ± 4.0	5.6 ± 1.9
Submaxillary	198 ± 22	150 ± 14	97.5 ± 16.1	6.1 ± 1.7

to that of a blank piece of paper. Each section was then placed in a counting vial, moistened with 0.25 ml of 1.0 N HCl and allowed to stand for 30 min. Ten ml of Bray's solution were then added for counting the efficiency under these circumstances being 4.7 per cent[a]

Results

The levels of tritiated NA retained in various mouse tissues 30 min after the injection of (±)-NA-7-³H are shown in Table I. These levels provide a measure of the ability of sympathetic terminals to take up NA into storage sites protected from metabolism. A striking decrease in the capacity to retain NA-³H was observed in the heart and submaxillary gland where 1.6 and 4.1 per cent respectively of the control levels of NA-³H were found. Immunosympathectomy was only partially effective in the intestine and the colon, where the NA retention was 33 and 47 per cent of normal respectively. Only a slight effect was observed with the mouse spleen. In contrast to the above tissues, immunosympathectomy almost doubled the retention of NA in the mouse vas deferens (Table I).

In these experiments a large dose (17.9 μg/kg) of NA was used in order to provide sufficient radioactivity in the tissues for accurate counting. It seemed possible that this large dose might distort the results because of disproportionate effects on blood flow to tissues with a limited vascular supply. Two immunosympathectomized mice and two controls were therefore injected with 1.79 μg/kg of the radioactive (±)-NA and tissue levels of total radioactivity and of NA-³H were measured (Table II). There was no significant change in the distribution of total radioactivity among the different organs. The relative effects of IS on NA-retention was changed in the

[a] More recent studies have shown that better resolution of NM and A can be obtained by substituting for the solvent of Roberts (1962) a mixture containing 2 parts of 0.2 M ammonium acetate [illegible] ...ining of the acidified paper strips has also [illegible] ...xture containing 4 g of 2,5-Bis 5-tertbutyl [illegible] ...iba Chemical and Dye Co. Fairlawn N.J. [illegible] ...an 1600 ml of toluene

TABLE II Retention of total radioactivity and NA-³H as in Table I after injection of (±)-NA-7-³H (1 79 µg/kg) Each value is the average of two animals

	µ µmoles, of labeled compound per g of tissue			
	Normal		Immunosympathectomized	
Organ	Total	NA-³H	Total	NA-³H
Vas deferens	8 3	3 2	10 0	4 0
Colon	9 8	4 8	6 6	3 1
Intestine	8 2	2 7	7 0	1 3
Spleen	3 2	2 0	3 1	1 6
Heart	44 7	41 0	2 8	1.2
Submaxillary	18 8	16 7	13 6	7 7

submaxillary gland and vas deferens but not in other organs It would appear that the absolute amount of NA-³H retained by the submaxillary of the IS mouse is independent of dosage The uptake of total radioactivity and NA-³H in vas deferens was now only slightly higher in IS mice compared to controls

The distribution of radioactivity among the metabolites of NA was measured in several tissues from normal and immunosympathectomized mice The heart was selected as one of the organs most responsive to IS and the vas deferens as one of the resistant The latter was also of interest because of the unexpectedly large uptake in the immunosympathectomized mouse

The paper chromatographic technique was selected in the hope of finding a less time-consuming procedure than that of Dengler *et al* (1962) for identification of metabolites by comparison of the distribution of radioactivity and ultraviolet absorption Fig 1 illustrates the analysis by ultraviolet absorption of a chromatogram obtained with NA, A, NM and M In Fig 2 the radioactive components in submaxillary glands of IS mice after NA-³H administration are seen to agree most closely with NA and NM This was also true for the the normal gland Radioactivities from the chromatogram of the latter are also plotted in Fig 2, but without the U V absorption data



resolution of these two compounds cannot be attained because of the relatively close R_f values obtained on companion strips. With mouse tissues the ethyl acetate extracts of the alumina eluates yielded only small fractions (less than 2 per cent) of the total radioactivity indicating that if any

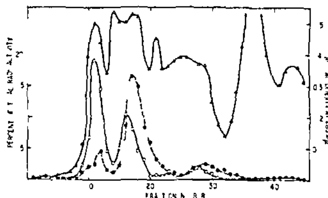


Fig 2 Chromatography as in Fig 1 of TCA extractable radioactivity from mouse submaxillary glands 30 min after i v injection of (±) NA 7 ³H (500 μC/kg) ○ Per cent of total cpm per 0 9 cm section of chromatogram from normal mouse ● Per cent of total cpm per 0 9 cm section of chromatogram from immunosympathectomized mouse △ Absorption at 280 mμ of sections of chromatogram from immunosympathectomized mouse

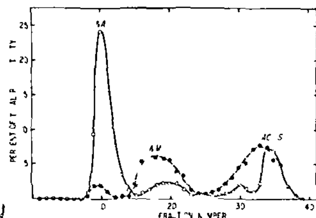


Fig 3 Chromatography as in Fig 1 and 2 of TCA extractable radioactivity from rat submaxillary ○ Control ● Immunosympathectomized

appreciable amounts of acidic metabolites were present they must have been methylated In the vas deferens however an accurate estimate of the percentage of these metabolites cannot be made due to the low number of total counts found in this small organ

In the submaxillary glands of both the immunosympathectomized mouse (Fig 2) and rat (Fig 3) the radioactivity retained after injection occurred mostly in the form of metabolites NM was predominant in the mouse while both NM and acid metabolites were significant components in the rat gland Oxidation to acid metabolites was also more extensive in the rat heart than in the mouse when the distribution of ³H was examined chromatographically Fig 4 and 5 illustrate representative experiments

The distribution of radioactivity among various metabolites in the pooled vas deferens of the immunosympathectomized mice was qualitatively different from that in the normal organ (Fig 6) Since only one chromatographic analysis of vas deferens was performed it is uncertain whether the increased proportion of the unknown metabolite in fractions 28 to 32 can be considered as a consequence of immunosympathectomy

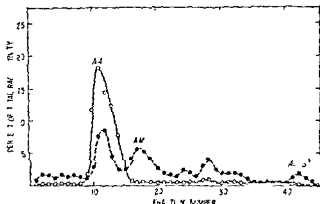


Fig 4 Chromatography as in Fig 1 and 2 of TCA extractable radioactivity from mouse hearts O, Control ●, Immunosympathectomized

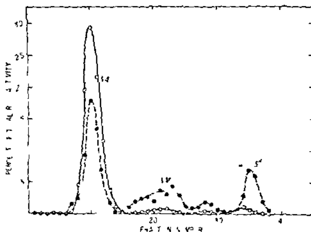


Fig 5 Chromatography as in Fig 1 and 2 of TCA extractable radioactivity from rat hearts ○, Control ●, Immunosympathectomized

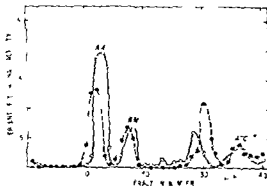


Fig 6 Chromatography as in Fig 1 and 2 of TCA extractable radioactivity from mouse vas deferens Organs from 4 animals were pooled to provide material for each chromatogram Control ● Immunosympathectomized

TABLE III Calculated NA metabolites retained after injection of (±)-NA-7-³H (17.9 µg/kg and 1.79 µg/kg). Each value is obtained from the mean values found in the previous tables

	µ µmoles of labeled NA metabolites per g of tissue			
	17.9 µg/kg		1.79 µg/kg	
Organ	Normal	Immunosympathectomized	Normal	Immunosympathectomized
Vas deferens	18.1	43.8	5.1	6.0
Colon	25.4	38.2	5.0	3.5
Intestine	13.4	67.4	5.5	6.7
Spleen	17.3	19.5	1.2	1.5
Heart	34	19.0	3.7	1.6
Submaxillary	18	91.4	2.1	5.9

Discussion

It would appear from the foregoing data that treatment with nerve growth factor antiserum is a reliable procedure for sympathetic denervation of heart and submaxillary gland, partially effective in colon, intestine and spleen but without effect in vas deferens. Histochemical studies by Hamberger *et al* (1964, 1965) first demonstrated that adrenergic neurons located in different body sectors of the rat responded differently to immunosympathectomy. They found virtually complete loss of noradrenaline in sympathetic nerves to the heart, submaxillary gland, iris and skeletal muscle vessels, partial loss of noradrenaline in adrenergic nerves to the intestine and intact noradrenaline store in vas deferens. Essentially similar findings have recently been obtained in the I.S. mouse (Levi-Montalcini *et al* 1967). The electronmicroscopic observations by Richardson (to be published) are in accord with these observations. Moreover, the ability of the heart to bind labeled NA in subcellular particles is lost after IS (Sjoqvist *et al* 1965).

Our data on the retention of NA in different IS tissues are therefore in good agreement with the quoted histochemical and electronmicroscopic observations. The relatively high uptake of NA in small intestine and colon of IS mice occurs in all probability in adrenergic nerve terminals which are resistant to IS. Part of the uptake may possibly take place in tissue cells belonging to the enterochromaffin system, since an increased number of such elements has been found in I.S. rats (Hamberger *et al* 1965), although a species difference may exist in this respect between rats and mice.

Morphological changes can hardly explain the increased uptake of NA in vas deferens of IS mice, an observation which has been made also in the rat by Zaimis *et al* (1965) and Iversen *et al* (1966). On the contrary, the electronmicroscopic observations revealed a normal distribution of the presumed subcellular storage particles for NA in this organ (Richardson, to be described in Levi-Montalcini *et al* 1967). It seems possible that exclusion of NA from larger organs may increase

the blood levels of NA-^{3}H and thus divert a bigger proportion of the amine to organs unaffected by immunosympathectomy

It is apparent from Table III that I S has little effect on the retention of NA metabolites in most of the tissues examined, although increases were noted in vas deferens and submaxillary gland These observations are in accord with the chromatographic evidence that metabolites, for the most part methylated, account for a larger percentage of the total ^{3}H in the extracts from the tissues of I S animals Iversen *et al* (1966) independently demonstrated an increased proportion of normetanephrine in the submaxillary gland and heart of I S rats following injection of NA These observations tally well with the experiments by Potter *et al* (1965) who found an increased content of O methylated metabolites of exogenous NA in the surgically denervated heart O Methylation thus appears to occur extraneuronally as might be expected from other studies showing that catechol O methyl transferase is extraneuronal (Kopin and Gordon 1963)

The I S submaxillary gland, where denervation is essentially complete, seems able to retain a small absolute amount of NA ^{3}H which does not vary as the dose of labeled NA is changed Insufficient I S material was available for a pharmacological analysis (cf Fischer *et al* 1965) of the site of this small NA retention Interestingly, extraneuronal binding of NA has been demonstrated histochemically to occur in the parenchyma of the submaxillary gland (Hamberger, Norberg and Olsson 1966)

Immunosympathectomized animals provide an excellent means of studying the role of sympathetic nerve endings in the uptake and metabolism of noradrenaline, but it is important to ascertain optimal effects of the antiserum treatment by suitable morphological and histochemical checks This is especially true, since a decrease in monoamine stores in one tissue compartment may be accompanied by an increase of monoamines in another compartment

Skillful technical assistance by Miss Patricia M Roddy is gratefully acknowledged

4

References

ANTON A H and D F SAYRE A study of the factors affecting the aluminum oxide — trihydroxy indole procedure for the analysis of catecholamines *J Pharmacol exp Ther* 1962 *138* 360—375

BRAY G A A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter *Analyt Biochem* 1960 *1* 279—285

[illegible]

HAMBERGER [illegible] d stribut[illegible]

HAMBERGE[illegible] sympath[illegible]

HAMBERGER B K A NORBERG and I OLSSON Extraneuronal binding of catecholamines and 3 4-dihydroxyphenylalanine (DOPA) in salivary glands *Acta physiol scand* 1967 *69* 1—12

HERTTING G and J AXELROD Fate of tritiated noradrenaline at the sympathetic nerve endings. *Nature* (Lond) 1961 *192* 172—173

HERTTING G, J AXELROD, I J KOPIN and L G WHITBY, Lack of uptake of catecholamines after chronic denervation of sympathetic nerves *Nature* (Lond) 1961 *189* 66

IVERSEN L L, J GLOWINSKI and J AXELROD The physiologic disposition and metabolism of norepinephrine in immunosympathectomized animals *J Pharmacol exp Ther* 1966 *151* 273—284

IVERSEN L L, J GLOWINSKI and J AXELROD, Reduced uptake of tritiated noradrenaline in tissues of immunosympathectomized animals *Nature* (Lond) 1965 *206* 1222—1223

KLINGMAN G I Catecholamine levels and dopa decarboxylase activity in peripheral organs and adrenergic tissues in the rat after sympathectomy *J Pharmacol exp Ther* 1965 *148* 14—21

KOPIN I J and E GORDON Metabolism of administered and drug released norepinephrine-7-H^3 in the rat *J Pharmacol exp Ther* 1963 *140* 207—216

LEVI-MONTALCINI R Growth control of nerve cells by a protein factor and its anti-serum *Science* 1964 *143* 105—110

LEVI-MONTALCINI R and P U ANGELETTI Growth control of the sympathetic system by a specific nerve growth factor *Quart Rev Biol* 1961 *36* 99—108

LEVI-MONTALCINI R and P U ANGELETTI Noradrenaline and monoamine oxidase content in immunosympathectomized animals *Int J Neuropharmacol* 1962 *1* 161—164

LEVI-MONTALCINI R and B BOOKER Destruction of the sympathetic ganglia in mammals by an antiserum to the nerve growth factor *Proc Nat Acad Sci* (Wash) 1960 *46* 384—391

LEVI-MONTALCINI R and S COHEN In vitro and in vivo effects of a nerve growth stimulating agent isolated from snake venom *Proc Nat Acad Sci* (Wash) 1956 *42* 695—699

LEVI-MONTALCINI R and S COHEN Effects of the extract of mouse submaxillary salivary gland on the sympathetic system of mammals *Ann N Y Acad Sci* 1960 *85* 324—341

LEVI-MONTALCINI R, K A NORBERG, L OLSON, K C RICHARDSON and F SJÖQVIST, Adrenergic nerves in immunosympathectomized animals To be publ 1967

POTTER L T, T COOPER, V L WILLMAN and D F WOLFE Synthesis binding release and metabolism of norepinephrine in normal and transplanted dog hearts. *Circulat Res* 1965 *16* 468—481

RICHARDSON K C The fine structure of autonomic nerve ending in smooth muscle of rat vas deferens *J Anat* (Lond) 1962 *96* 427—442

ROBERTS M A note on the use of cellulose phosphate cation exchange for the separation of catecholamines and some other biogenic amines *J Pharm Pharmacol* 1962 *14* 746—749

SJÖQVIST F, F TITUS, I A MICHAELSON, P W TAYLOR Jr and K C RICHARDSON Uptake and metabolism of d l norepinephrine-7-H^3 in tissues of immunosympathectomized mice and rats. *Life Sci* 1965 *4* 1125—1133

ZAIMIS E, L BERK and B A CALLINGHAM Morphological biochemical and functional changes in the sympathetic nervous system of rats treated with nerve growth factor antiserum *Nature* (Lond) 1965 *206* 1220—1222

Acta physiol scand 1967 69 23—28

From the Surgical Research Laboratory Thoracic Clinic, Karolinska Sjukhuset Stockholm Sweden

The Effect of Cholecystokinin-Pancreozymin Preparations on Hepatic Bile Output in Fasting and Digesting Dogs

By

G Jonson, G Svartengren and L Thulin

Received 17 June 1966

Abstract

Jonson G, G Svartengren and L Thulin *The effect of cholecystokinin pancreozymin preparations on hepatic bile output in fasting and digesting dogs* Acta physiol scand 1967 *69* 23—28

decrease These patterns appear to be related to the gastric and duodenal activity during the experiments Physiologic or pharmacologic interventions on hepatic bile output should thus be evaluated only when side effects on gastro-intestinal motility and secretion can be excluded

Friedman and Snape (1945) found that all their extracts from intestinal mucosa increased the bile secretion in the dog, whereas only some of them increased the secretion of pancreatic juice They suggested a hormone, "hepatocrinin", which specifically stimulates the bile secretion

Edholm, Jonson and Thulin (1962) found that a preparation of cholecystokinin pancreozymin had a choleretic effect in the fasting dog apart from the effect of the secretin contained therein Debray *et al* (1963) found that a preparation of pancreozymin had a similar effect in the rat

The present study was made in order to evaluate the effect on the bile output of more active preparations of cholecystokinin pancreozymin in the fasting and the digesting dog

Procedure

from the cholecystokinin. With the infusions used the secretin dose never exceeded 1 clinical unit per kg body weight and hour which is below the threshold for choleretic effect (Jonson *et al* 1964)

TABLE I. Fasting, digestion of food with bile, infusion of cholecystokinin-pancreozymin and of hydrochloric acid (0.5 g per kg b. w. a. hr) in 3 dogs. Procedure

Expt	Dog	Preparat. Dose. Ivy dog units per kg b. w. per hr	Experimental conditions	Time hrs (0–9)
1	A		Fasting	0–8
2	B		Fasting	0–8
3	A		Digestion	2–8
4	B		Fasting	0–2
5	A	PTEAE 1162 0.5	Hormone	2–4
6	B	PTEAE 1162 0.5	Fasting	0–8
7	B	FeCr II 0.5	Hormone	4–6
			Fasting	0–8
8	A	PTEAE 1162 0.5	Hormone	4–6
9	A	PTEAE 1162 0.5	Digestion	2–8
10	B	FeCr II 0.5	Fasting	0–2
11	C	Commercial Cecekin 10.0	Hormone	1–2; 5.5–7
			Digestion	3–8
12	C		Fasting	0–3
13	C	Commercial Cecekin 10.0	Hormone	1–2; 5–7
			Dehydrocholic acid	3–8.5
			Fasting	0–9

Three adult mongrel dogs were cholecystectomized and provided with a permanent external two-part common duct fistula (Jonsson 1963). At the same time each day they consumed a standard meal mixed with all the bile produced during the previous 24 hrs. The concentration of bilirubin and the activity of alkaline phosphatase in serum was normal and the daily bile output was constant (standard deviation less than ... per cent).

Before each experiment, which started at ... a.m., the dogs had been fasting for 24 hrs. They were placed in a Pavlov frame and were unanaesthetized. The outer end of the fistula was placed at the level of the xiphoid process.

In 9 expts. the dogs were given infusions of intestinal hormones during fasting, during digestion of food with bile and during infusion of dehydrocholic acid. In 4 expts. the bile output was studied during fasting or during digestion (Table I).

The bile produced was measured at 30 min intervals in exp. 1–10 and at 15 min intervals in exp. 11–13. The bile of transitional stages, i.e. the first 15 or 30 min of each experimental period was omitted. The bile output was calculated as the mean of the measured volumes and was expressed as multiples of the initial output, i.e. the output during the first fasting period. In exp. 13 the output during infusion of dehydrocholic acid increased slowly for 90 min and then remained for 45 min at a constant level which was considered to be the bile output under influence of dehydrocholic acid.

TABLE II Output of hepatic bile during fasting and digestion No hormonal infusion

	Exp no	Bile output (multiples of initial)		
		2nd 2 hr period	*3rd 2 hr* period	*4th 2 hr* period
Fasting	1	0 9	0 8	0 8
	2	1 4	1 3	0 7
Digestion	3	3 2	3 3	3 7
	4	4 4	4 0	4 2

TABLE III Output of hepatic bile during and after infusion of hormonal preparations in fasting dogs

Exp no	Bile output (multiples of initial)		
	During infusion	After infusion	
5	2 4	1 1	0 8
6	2 1	0 8	0 8
7[1]	2 4	0 9	
11	3 0	1 1	
12	1 8	0 9	
13	1 8	1 2	

[1] In this experiment there was a 2 hr interval between the initial period and the infusion The output during that period was equal to the initial

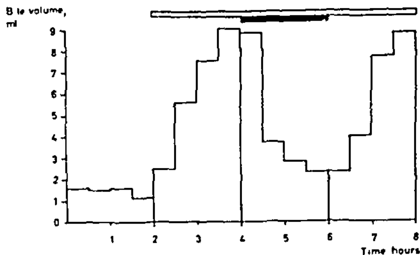
TABLE IV Effect of infusion of hormonal preparations during digestion of food with bile or during infusion of dehydrocholic acid

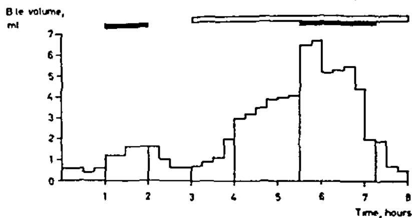
Exp no	Bile output (multiples of initial)		
	Before	During	After
8	3 8	3 2	4 4
9	2 9	2 4	3 3
10	5 0	2 0	4 7
11	5 3	8 4	0 9
12	4 2	7 1	0 9
13[1]	5 9	6 8	6 9

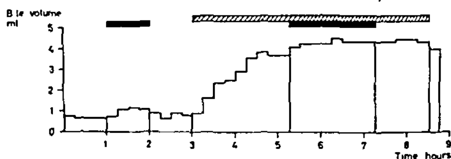
[1] Infusion of dehydrocholic acid (0 5 g per kg b w per hr) instead of digestion of food with bile

Results

The initial bile output in dog A was 3 2—4 4 ml per 30 min (5 expts , mean 3 7), in dog B 1 0—1 8 ml (5 expts , mean 1 4) and in dog C 1 1—1 4 ml (3 expts , mean 1 3)







▭ = Food w th b le   ▬ = Cholecystokinin– pancreozymin

▨ = Dehydrocholic acid

During continued fasting without infusion, the bile output was about equal to the initial value During digestion without infusion the bile output was 3—4 times the initial value (Table II)

In the fasting dogs, the bile output increased during hormonal infusion up to 2—3 times the initial value After infusion the output returned to the initial value (Table III)

In the digesting dogs, the hormonal infusion had a varying effect (Table IV) in some experiments the mean output decreased, whereas in other in increased In exp 8, 9 och 10 the output did not change during the first 30 min of infusion, but then it decreased to a lower level (Fig 1, upper) In exp 11 and 12 the output increased markedly during the first 60 min of infusion and then gradually decreased (Fig 1, middle)

During infusion of dehydrocholic acid in the fasting dog, the bile output was approximately 6 times the initial value During and after superimposed hormonal infusion, the output increased further by about 15 per cent (Table IV, Fig 1, lower)

Preliminary analyses of the bile were made in exp 5 and 6 During infusion of cholecystokinin pancreozymin in the fasting dog the concentration of total dry matter decreased by approximately 50 per cent The biliary concentration of bilirubin and activity of alkaline phosphatase decreased parallel to the concentration of the total dry matter, while the concentration of phospholipids decreased by about 80 per cent The changes in the composition of the bile will be further studied

Discussion

Earlier findings of a choleretic effect in fasting dogs of cholecystokinin pancreozymin preparations, apart from the secretin contained therein, were confirmed The effect may be due to the cholecystokinin pancreozymin itself and/or other substances Cholecystokinin preparations are known to influence the gastric and duodenal motility (Adlercreutz *et al* 1960, Torsoli *et al* 1961, Johnson *et al* 1965) and also the gastric acid secretion (Gillespie *et al* 1964) As digestion in these fistula dogs means digstion of food and bile taken orally, the gastric emptying rate is of primary importance to the bile output The output curves (Fig 1) then suggest that there was a reduced gastric emptying in exp 8, 9 and 10 with the preparations PTEAE 11462 and PcC II but a fast and complete emptying in exp 11 and 12 with the commercial preparation 'Cecekin"

In exp 13, the infusion of dehydrocholic acid replaced the digestion of food with bile and eliminated any influence of gastric motility on the bile output The same dog and the same preparation was used in exp 11, 12 and 13 That the output did not decrease under these conditions, proved that the bile output patterns in the digestion experiments depended on the hormonal effect on gastric motility

Thus the effect of physiologic or pharmacologic interventions on hepatic bile output can be evaluated only when side effects on gastro-intestinal motility can be excluded In general, pharmacologic effects should preferably be studied only during fasting

Supported by grants from the Gustaf and Tyra Svenssons memorial Fund, Stockholm.

References

ADLERCREUTZ, E., E. PETTERSSON, H. ADLERCREUTZ, P. GRIBBE and C. WEGELIUS: Effect of cholecystokinin on duodenal tonus and motility. *Acta med. scand.* 1960, *167*, 339—342.

DEBRAY, CH., CH. VAILLE, J. DE LA TOUR, CL. ROZE, MLLE M. SOUCHARD: La pancréozymine. Etat actuel de la question. *Rev. inter. d'hepat.* 1963, *13*, 473—490.

EDHOLM, P., G. JONSSON and L. THULIN: Le debit biliaire du foie. Stimulation par la cholecystokinine, la sécrétine et l'alimentation per-orale. *Path. et Biol.* 1962, *10*, 447—450.

FRIEDMAN, M. H. F. and A. J. SNAPE: Comparative effectiveness of extracts of intestinal mucosa in stimulating the external secretions of the pancreas. *Fed. Proc.* 1945, *4*, 21—22.

GILLESPIE, IAIN E. and MORTON I. GROSSMAN: Inhibitory effect of secretin and cholecystokinin on Heidenhain pouch responses to gastrin extract and histamine. *GUT* 1964, *5*, 342—345.

JOHNSSON, LLOYD P. and DONALD F. MAGEE: Inhibition of gastric motility by a commercial duodenal mucosal extract containing cholecystokinin and pancreozymin. *Nature* (Lond.) 1965, *207*, 1401.

JONSSON, G.: Permanent biliary fistulas in the dog. *Acta chir. scand.* 1963, Suppl. 316.

JONSSON, G., L. SUNDMAN and L. THULIN: The influence of chemically pure secretin on hepatic bile output. *Acta physiol. scand.* 1964, *62*, 287—290.

TORSOLI, A., MARIA LETIZIA RAMORINO, L. PALAGI, G. COLAGRANDE, L. BASCHIERI, S. RIBOTTA and M. MARINOSCI: Observations roentgen cinématographiques et électromanométriques sur la motilité des voies biliaires. *Sem. Hôp. Paris* 1961, *37*, 790.

Acta physiol. scand. 1967. 69. 29—36

From the Department of Physiology, Veterinarhogskolan, Stockholm, Sweden

A Study of Thirst and Other Effects of an Increased Sodium Concentration in the 3rd Brain Ventricle

By

B Andersson, M Jobin[1] and K Olsson

Received 20 June 1966

Abstract

Andersson, B , M Jobin and K Olsson *A study of thirst and other effects of an increased sodium concentration in the 3rd brain ventricle* Acta physiol scand 1967 *69* 29—36

The characteristics of thirst elicited by injections of hypertonic NaCl solution into the 3rd brain ventricle were studied in goats supplied with permanent ventricular cannulae Animals in normal water balance drank 1 5 to 2 0 l of water within 2 min after the intraventricular injection of 0 1 ml of 0 85 M NaCl In the hydrated animal this thirst response was markedly diminished or completely [illegible]

inhibit temporarily an established water diuresis Aldosterone treatment did not prevent the increase in urinary electrolyte excretion occurring after injections of hypertonic NaCl into the 3rd ventricle

Repetitive injections of 0 1 ml of 0 85 M NH_4Cl or of 1 7 M d glucose into the 3rd ventricle of injections of 0 1 ml of 0 85 M NaCl into the lateral ventricle did not induce drinking or cause any increase in urinary electrolyte excretion

Injections of small amounts of hypertonic NaCl-solution into, or in the vicinity of the 3rd ventricle of the brain have earlier been found to elicit polydipsia in the goat (Andersson 1953) For this and other reasons it has been suggested that a hypothalamic 'osmoreceptor" mechanism (similar to, or identical with that shown by Verney (1947) to control the secretion of antidiuretic hormone) may be of importance for the development of the urge to drink

In this paper is given an account of experiments in which the use of chronic preparations has provided possibilities to study further the characteristics of thirst elicited by injections of hypertonic NaCl into the 3rd ventricle of the brain As reported in a preliminary communication (Andersson, Jobin and Olsson 1966 a), such injections were further found to cause increased urinary salt excretion The results of a continued study of this phenomenon is also reported below

[1] Postdoctoral Research Fellow N R C Canada From the Department of Physiology, Medical School Laval University Quebec P Q

Methods

Four female goats (b wt about 40 kg) were used The animals had cannulae permanently implanted into the 3rd ventricle of the brain. One of the goats had an additional cannula placed in the right, lateral ventricle The techniques of implantation and injection were described earlier (Andersson Jobin and Olsson 1966 b) The use of permanent cannulae made it possible to make repeated experiments during several (5 to 8) months in each animal The effects of intraventricular injections could therefore be studied in the same goat both during periods when no supplementary sodium was added to the diet, and during periods when the animals received 6 or 10 g NaCl added to the daily food ration

During all experiments the animals remained in their usual environment in metabolism cages In order to avoid the stress of catheterization and the irritation of a retention catheter present in the urinary bladder the urine was collected when spontaneously voided Blood samples were taken [illegible]

Results

A Thirst

I Injections into the 3rd ventricle of the brain

a) 0 85 M NaCl-solution Before any intraventricular injection was performed, the goats had always free access to water and could therefore be expected to be in normal water balance In most experiments only about 100 ml of water was left available to the animals in the bottom of the pail at the time of injection Under these circumstances the injection of 0 1 ml of 0 85 M NaCl into the 3rd ventricle invariably caused a strong urge to drink within 30 to 90 sec The injections were not seen to have any other behavioural effects and did not cause any visible irritation to the animals The goats drank the small amount of water available, and then eagerly licked the bottom of the pail or looked around for more water Repeated tests with negligible amounts of water revealed that the urge to drink gradually became weaker and disappeared in about 20 min On renewed injections at this stage the urge to drink reappeared with full strength and with shorter latency time (10 to 15 sec) than after the first injection

In experiments in which the goats had free access to water also after the injection of 0 1 ml of 0 85 M NaCl, the animals drank in one sequence 1 5 to 2 0 l of water within 2 min after the injection Occasionally a small amount of water was drunk again a few min later When in this manner the goats had been allowed to quench their thirst after a first injection of hypertonic NaCl, a renewed injection, performed 20 min after the first gave a much reduced drinking response This inhibitory effect of hydration was even more obvious when the goats were hydrated by giving 5 l of water by stomach tube 1 1/2 hr before the injection of hypertonic NaCl Then, no drinking at all occurred as a result of the injection

b) Other hypertonic solutions In an attempt to determine whether the drinking effect might be attributed either to the sodium or to the chloride ion, hypertonic solutions of other salts were injected into the 3rd ventricle The results of injections of sodium salts with other anions than Cl were inconclusive A first injection of 0 1

ml of 0 85 M Na lactate induced an obvious urge to drink, but this was not the case with similar injections performed 30 min later However, repetitive injections of this salt caused trembling and excitation Even more pronounced disturbing effects (weakness and sluggishness) were seen soon after test injections of 0 1 ml of 0 85 M $NaHCO_3$ which gave no clear drinking response

More conclusive results were obtained from injections of 0 1 ml of 0 85 M NH_4Cl and of 1 7 M d glucose These injections did never induce any visible urge to drink in the goats and were not seen to disturb the animals Moreover, injections of 0 1 ml of 0 85 M NaCl performed 5 to 15 min later gave the usual thirst effect, indicating that no side effects of NH_4Cl or glucose had been the reason for the negative drinking response to these substances

2 *Injections into the lateral ventricle of the brain*

Comparisons between the effects of injections of 0 1 ml of 0 85 M NaCl into the lateral ventricle and into the 3rd ventricle were made on several occasions in one of the goats In contrast to the injections into the 3rd ventricle, the injections of this amount of hypertonic NaCl into the lateral ventricle did not elicit drinking

B Urine flow and electrolyte excretion

1 Injections into the 3rd ventricle

a) Hypertonic NaCl solutions Studies of the effects of injections of hypertonic NaCl on urine flow and urinary excretion of electrolytes were started during a period when the animals received no supplementary NaCl and therefore had a low basic urinary sodium excretion (5 to 10 μeq/min) and a low urinary sodium concentration (5 to 30 meq/l) Under such conditions relatively high hematocrit and plasma protein values were seen indicating the presence of a certain hemoconcentration

Injection of 0 1 ml of 0 85 M NaCl were performed at 30 min intervals for periods of 2 hrs and the animals were not allowed to drink during the injection period and the following 3 hrs As a result of the injections the urinary Na and Cl excretion rose markedly showing a 5 to 10 fold increase over pre injection level towards the end of the injection period The K^+ excretion was approximately doubled and urine flow increased by 2 to 3 times The urinary electrolyte excretion remained at this high level for about one hr after the last injection Then it fell to pre injection level concomitant with a gradual decrease in urine flow A second and more pronounced diuresis (up to 7 times basic flow) usually occurred about 2 hrs after the last intra ventricular injection of hypertonic NaCl This second increase in urine flow had the character of a water diuresis (salt excretion remaining at or below basic level) while urinary electrolyte concentration dropped to very low levels A 5 to 10 % decrease in hematocrit and plasma protein was usually observed during the period of intra ventricular injections when the goats received no extra NaCl

When the animals received supplementary NaCl in the diet, the relative effects of a similar sequence of injections of hypertonic NaCl were the same However, the

absolute increase in Na and Cl excretion in these experiments was much greater In some experiments the Na excretion rose from a basic level of less than 100 µeq/min to more than 1000 µeq/min (Fig 2, left above) At the same time urinary Na concentration rose above 200 meq/l Determinations of hematokrit and plasma protein indicated that the addition of NaCl to the diet by itself had caused a 10 to 15 per cent expansion of the blood volume in the goats This may be the reason why a fall in hematokrit and plasma protein was less consistently observed due to the intraventricular injections when the animals were on a diet rich in NaCl

In an attempt to determine the threshold for the effect on urinary electrolyte excretion some experiments involving single intraventricular injections of 0 2 ml of 0 85 M NaCl or repetitive injections of 0 1 ml of less hypertonic NaCl were performed The single injections caused a similar but more short lasting increase in urinary Na and Cl excretion Maximal excretion rate occurred about one hr after the injection and the electrolyte excretion had returned to basic level about one hr later When 5 injections of 0 1 ml of 0 51 M NaCl were made during a 2 hr period the effect on the urinary excretion of Na and Cl was about 50 per cent of that obtained with a similar sequence of injections of 0 85 M NaCl in the same animal Repetitive injections of 0 1 ml of 0 34 M NaCl gave a response that was only about 20 of the response to 0 85 M NaCl

Determinations of blood glucose revealed no change or a minor increase (up to 15 per cent during the periods of intraventricular injection of hypertonic NaCl No consistent change in plasma Na and K was observed but plasma Cl usually fell by about 5 per cent during the injection period and returned to preinjection level about 2 hrs later

Single and repetitive injections of 0 1 ml of 0 85 M NaCl were also made during water diuresis The goats had then received 4 to 5 l of water 1 1 2 hr prior to the first injection of hypertonic NaCl After a single injection the diuresis was reduced to half or one third of the preinjection level within 30 min and then rose again during the following hr A similar but more long lasting inhibition of water diuresis occurred during the periods of repetitive injections of hypertonic NaCl The urinary concentration of electrolytes rose considerably during the inhibition of water diuresis resulting in a net increase in Na and Cl excretion of the same order as that seen in the not hydrated goat due to similar injections The observed inhibition of water diuresis indicated that the injection of small amounts of hypertonic NaCl into the 3rd ventricle caused a significant release of antidiuretic hormone In order to determine whether an excessive release of antidiuretic hormone might be the cause of the observed increase in urinary electrolyte excretion the effect of repetitive intravenous injections of antidiuretic hormone Pitressin Parke Davis et Co) was studied The injections of 20 or 100 mU of pitressin every 30 min during periods of 2 hrs did not cause any significant change in the electrolyte excretion of the goats

In an attempt to find out if the observed increase in urinary Na and Cl excretion might be the result of an inhibition of aldosterone secretion repetitive injections of 0 1 ml of 0 85 M NaCl into the 3rd ventricle were made in two of the goats when

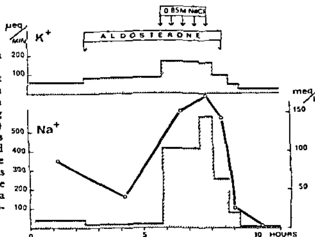


Fig. 1. Effect on urinary excretion of K and Na of a series of 5 injections of 0.1 ml of 0.85 M NaCl at 30 min intervals into the 3rd brain ventricle during treatment with aldosterone. I.v. injections of 25 μg of aldosterone (Aldocorten, Ciba) were made every 30 min. This treatment started 3 hrs before and was continued for one hr after the period of intraventricular injections of NaCl. The double lined curve shows the changes in urinary Na concentration. (Goat's diet supplemented with NaCl.)

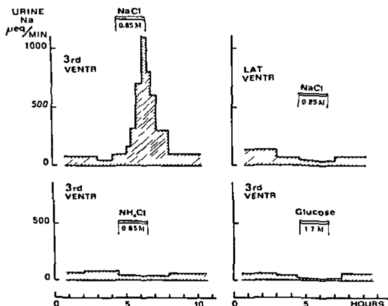


Fig. 2. Urinary Na excretion during repetitive injections of hypertonic solutions into the brain ventricular system. (Goats receiving supplementary salt in the diet.)
Upper left: Stimulation of Na excretion by a series of 5 injections of 0.1 ml of 0.85 M NaCl at 30 min intervals into the 3rd ventricle.
Upper right: Lack of effect of this sequence of 0.85 M NaCl injections into the lateral ventricle of the same goat.
Lower left and *right*: Absence of response to repetitive injections of 0.1 ml of 0.85 M NH_4Cl or 1.7 M d-glucose injections into the 3rd ventricle (5 injections with 30 min intervals).

exogenous aldosterone (Aldocorten, Ciba") was given i.v. to the animals. The period of aldosterone treatment (dose: 25 μg every 30 min) preceded the intraventricular injections by 3 hrs and was continued for one hr after the last intraventricular injection. Although the basic urinary Na excretion was depressed by the al-

the intraventricular injections of hypertonic NaCl still caused the usual conspicuous increase in Na and Cl excretion and a considerable rise in the urinary concentration of these ions (Fig 1)

b) Other hypertonic solutions Urinary electrolyte excretion was also followed in experiments involving repetitive injections of 0 1 ml of 0 85 M NH_4Cl and of 1 7 M d glucose into the 3rd ventricle As illustrated in Fig 2 such injections having a negative drinking effect did not cause any increase in urinary electrolyte excretion either In a preliminary experiment, 0 2 ml of 1 7 M glucose was injected into the 3rd ventricle of a hydrated goat This was not seen to cause any inhibition of the water diuresis

2 *Injections into the lateral ventricle*

For comparison repetitive injections (5 injections with an interval of 30 min) of 0 1 ml of 0 85 M NaCl were made either in the lateral or in the 3rd ventricle of one of the goats No increase in urinary electrolyte excretion and urine flow was observed when the injections were made into the lateral ventricle The different responses of the same animal to identical injections of hypertonic NaCl into the 3rd ventricle and into the lateral ventricle are shown in Fig 2 (*above*)

Discussion

A change in the internal environment leading to cellular dehydration elicits thirst together with an increased secretion of antidiuretic hormone from the neurohypophysis Verney s 1947) refined experiments in the dog have revealed that the latter response is mediated by a central osmoreceptor mechanism By intracranial vascular ligations it has been possible to localize these "osmoreceptors in the anterior hypothalamus Jewell and Verney 1957) Apparently the "osmoreceptors are not stimulated by a rise in total body fluid osmolarity *per se* but rather by changes in the extracellular fluid which reduce the cell volume such as an elevated Na^+ concentration in the blood Thus Verney 1947 has shown that intracarotid infusions of hypertonic Na salts are much more effective in eliciting a release of antidiuretic hormone than infusions of hypertonic K salts urea or glucose which are transferred much more readily into the cells

In earlier acute experiments it was found that injections of small amounts of hypertonic NaCl into the anterior medial hypothalamus or into the 3rd ventricle may elicit drinking in the goat Andersson 1953) This effect however was not very reproducible on renewed injections and a nonspecific stimulatory effect of the hypertonic NaCl could not be excluded The present experiments provide more direct evidence that hypothalamic osmoreceptors in Verney s sense are concerned not only with the release of antidiuretic hormone but also with the development of the urge to drink Of the hypertonic solutions tested only the Na salts elicited thirst whereas the NH_4Cl and glucose which are likely to move more readily into the cells were negative The thirst response to injections of hypertonic NaCl into the 3rd ventricle was very reproducible when water was restricted but was inhibited

or blocked by voluntary overdrinking or forced hydration However, in the hydrated animal a temporary inhibition of water diuresis occurred, suggesting that the injections stimulated osmoreceptors regulating the secretion of antidiuretic hormone This stimulation is likely to have been considerably greater in the non hydrated goat That the hypothalamus is the responding part of the brain is indicated by the negative response to lateral ventricular injections of hypertonic NaCl

The present experiments do not afford any definite explanation for the mechanism responsible for the conspicuous increase in Na^+ and Cl excretion caused by the injections of hypertonic NaCl into the 3rd brain ventricle However, this response and the lack of response to lateral ventricular injections suggest the existence of some kind of feed back control of the Na^+ concentration of the extracellular fluid which is located near the 3rd ventricle Such a control does not seem to be effected through changes in aldosterone secretion since the increase in urinary salt excretion was of the same order in the aldosterone treated animal The possibility remains that it may involve a modification of the aldosterone effect on the renal tubular reabsorption of sodium

The inhibition of water diuresis obtained in the hydrated goat when the NaCl concentration was raised in the 3rd ventricle indicates that antidiuretic hormone release was stimulated Since repetitive injections of large doses of pitressin did not cause any increased urinary salt excretion, it seems unlikely that an augmented release of antidiuretic hormone should have caused the observed increase in urinary salt excretion If however, large amounts of antidiuretic hormone were released in this manner in response to repetitive 3rd ventricular injections of hypertonic NaCl in the not hydrated goat, there could be following the injection period, a temporary lack of hormone available for release This could possibly explain the occurrence of a high flow of very dilute urine about 2 hrs after the end of the injection period

It has earlier been observed that ephedrine and adrenaline injections cause a transient diuresis and a rise in urinary NaCl excretion in the goat and that these effects apparently are secondary to an increased glomerular filtration rate (Andersson 1955) It seems unlikely, however that the increased urinary salt excretion observed here should have been secondary to a non-specific adrenaline release The goats were not visibly irritated by the intraventricular injections of NaCl and no or only a minor rise in blood glucose was seen during the injection periods Furthermore, similar injections of hypertonic NH_4Cl or glucose were negative Still, at the present time one cannot exclude an increased glomerular filtration rate as the direct cause of the observed changes in urinary salt excretion A fall in hematocrit and plasma protein was observed in most cases during the periods of repetitive injections of hypertonic NaCl into the 3rd ventricle These changes in blood composition may have reflected an expansion of the blood volume or the total extracellular fluid volume leading to an increase in glomerular filtration rate It is hoped that future studies of body fluid distribution with more adequate methods shall reveal whether such an expansion of the blood or total extracellular fluid really takes place in response to a raised NaCl concentration in the 3rd ventricle

This work was supported by the Swedish Medical Research Council (Project No 14x 503-02)

References

ANDERSSON, B, The effect of injections of hypertonic NaCl solutions into different parts of the hypothalamus of goats *Acta physiol scand* 1953 *28* 188—201

ANDERSSON, B, Observations on the water and electrolyte metabolism in the goat *Acta physiol scand* 1955 *33* 50—65

[illegible]

BRUN C Microdeterminations of chlorides in blood *Nord Med* 1919 *42* 1774—1775

JEWELL P A and E B VERNEY, An experimental attempt to determine the site of the neurohypophysial osmoreceptors in the dog *Phil Trans B* 1957 *240* 197—324

LEVIN K and S LINDE Bestämning i glykos i blod, liquor och urin med ett nytt glykosoxidasreagens *Svenska Läk -Tidn* 1962 *59* 3016—3026

WEICHSELBAUM T E An accurate and rapid method for the determination of proteins in small amounts of blood serum and plasma *Amer J clin Path* 1946 *10* 40—49

VERNEY, E B The antidiuretic hormone and factors which determine its release *Proc roy Soc B* 1947 *135* 25—106

Acta physiol. scand. 1967. 69. 37—50

From the Department of Physiology, Karolinska institutet, Stockholm, Sweden

Application of High-Frequency Reflectometry for Measurement of Minute Changes in Electrolytic Conductance

By

L. HAAPANEN

Received 29 June 1966

Abstract

HAAPANEN, L. *Application of high frequency reflectometry for measurement of minute changes in electrolytic conductance.* Acta physiol. scand. 1967. 69. 37—50.

[illegible]

maximum occurs at conductivities around 10^{-4} ohm^{-1} cm^{-1}, at which a change in conductivity of 10^{-7} ohm^{-1} cm^{-1} can be measured with reasonable accuracy in long term experiments and transient changes of 10^{-8} ohm^{-1} cm^{-1} can be detected using variations in the capacitive component as index. By means of a graphical method an analysis is made of an equivalent circuit simulating the electrical properties of an electrolyte. It is shown that a series circuit of a varying resistance and a fixed value capacitance approximates several of the characteristics of a solution.

Introductory comments on conductivity measurements

The concentration of an electrolyte can be determined by measuring its electrical conductance which is the reciprocal of the resistance. Usually, alternating current in the low-frequency range, *e.g.* 1,000 cycles per sec, is applied to the solution by means of two electrodes mounted together to form a conductivity cell, and as a rule the measurement is performed by means of an audio-frequency variation of the four armed Wheatstone bridge. When electric current is passed through an electrolyte the solution behaves as if the conductance were associated with a capacitance, the importance of which grows with the measuring frequency (*cf.* section 3), and to obtain an exact balance of the Wheatstone bridge arrangements have thus to be made to compensate for the influence of this capacitance.

The commercially available conductivity cells are often designed to give optimum performance over specific ranges of conductivity. For electrolytes of low conductivity

are used large electrodes placed close together, for solutions of high conductivity small electrodes far apart A typical general purpose conductivity cell is usually designed for a sample volume of about one centimetre cube Such a cell is well suited for measurements on well stirred solutions of relatively large volumes, the whole volume of fluid being of the same ionic concentration

For measurements of local concentration variations, the standard conductivity cells are however often inadequate owing to their poor space resolving power, and the conductivity measured will be the result merely of the combined effect of many small volume elements of electrolyte, each of different concentration For such specific purposes it will thus be necessary to design special conductivity cells or probes Measurements of the concentration in a small volume element require that at least one of the electrodes should be of small size Lamb, Manning and Wilhelm (1960), using one electrode of small dimensions and a second large electrode located at some distance in the fluid showed that concentration measurements were possible in volume elements of the order of 3×10^{-4} ml However as the dimensions of one or both of the electrodes are reduced polarization may become a disturbing factor One method by which the effect of polarization can be counteracted is to increase the measuring frequency of the conductivity bridge, another is to reduce the current flowing through the electrodes in which case a more sensitive device must be used to detect the balance point of the bridge Most conductivity bridges are based on manual adjustment of the ratio arms and thus not suited for following rapid changes in conductance For measurements of dynamic local concentration variations it thus seems advantageous to move the measuring frequency up in the radiofrequency range *e g* 1 000 kilocycles per sec and utilize the specific effects observed when an electrolyte is inserted into an electrical field at these frequencies

An electrolyte exposed to a high frequency field absorbs energy in the same manner as a dielectric material of high losses and thus exerts a loading effect on the oscillator supplying the field It was shown by Forman and Crisp (1946) that when an electrolyte solution is placed in a field of a particular frequency the energy absorption reaches a maximum at a certain concentration of the solution If another frequency is chosen maximum absorption occurs at another concentration and the authors found that a simple empirical relation exists *viz*

$$\lambda \times \gamma = K_e$$

where λ = wavelength corresponding to the frequency

γ = concentration of solution for maximum power absorption

K_e = particular constant for each simple electrolyte

Within certain practical limits it is thus possible to choose an oscillator frequency resulting in maximum electrolytic power absorption at any desired concentration However, to a lesser extent power absorption will occur also for higher and lower concentrations

The main application for instruments utilizing the described effect has been the determination of titration end points A common method has been to place the vessel containing the test solution inside the inductor of the oscillator or between

the plates of a capacitor constituting part of its resonant circuit The vessel is made of low loss insulating material, usually glass, no special electrodes being required A change in ionic composition of the solution in the course of the titration results in a change in the parameters of the oscillator due to a variation in the energy absorption A plot of any of the currents or voltages or of the frequency of the oscillator against volume of standard solution is made At the end point of the titration a break in the resulting curve will be obtained A large number of high frequency titrimeters have been described (*cf*, *e g*, Blaedel and Malmstadt 1950, Hall 1952 b), most of which have been based on the measurement of one single oscillator parameter, either a current a voltage, or the frequency

The availability in this laboratory of an instrument originally designed for use as an extremely sensitive capacitance meter of the high frequency type (Haapanen 1962) initiated investigations as to the possibility of converting it into an instrument suited for studies of certain biological ionic events, utilizing the power absorbing effect of an electrolyte described above The principle of the original instrument is to determine capacitance changes by measuring the phase shift between two loosely synchronized high frequency oscillators, which makes the method highly sensitive With only minor modifications of the instrument one more parameter, such as the grid current can be measured simultaneously thus making the instrument more versatile

[illegible]

described

In the first section of this paper the principle of using the high frequency reflectometer for measurement of incremental changes in electrolytic conductance is illustrated by means of a block diagram from which it appears that changes in the resistive and capacitive components of the combined probe and electrolyte impedance are measured separately as variations in oscillator grid current and phase shift respectively In the second section quantitative data are given on the sensitivity of these two parameters to changes in conductivity In the third section a graphical analysis is made of a hypothetical equivalent circuit of an electrolyte

1 Working principles of the high frequency reflectometer

The principle of using the high frequency reflectometer for the measurement of conductivity changes appears from the block diagram in Fig 1 By means of a probe consisting of two platinum electrodes immersed in the electrolyte solution the high frequency voltage from the resonant circuit of a frequency variable oscillator is applied to the solution The electrolyte known to possess resistance and capacitance, is represented by the resistor R and the capacitor C within the circle to the left in the figure For reasons which will be explained below an external capacitor is connected

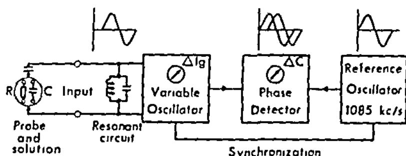


Fig 1 Block diagram of high frequency reflectometer See text

in series with one of the electrodes of the probe in all experiments unless otherwise stated

Increasing the concentration of the solution has the effect of decreasing the value of the resistor representing the solution and results also in a change in the capacitance of the solution The change in the resistive loading corresponding to an increased absorption of high frequency energy causes the oscillation amplitude to diminish, bringing about a decrement, ΔI_g, in the grid current (I_g, which is caused by rectification of part of the high frequency voltage across the resonant circuit) The sensitivity of the grid current to small changes in loading is due to the very loose coupling between the oscillator and its resonant circuit There is a limit to the minimum value of resistance, *e g* the maximum value of conductance that can be applied across the resonant circuit without extinguishing oscillations altogether

By placing known resistors in parallel with this resonant circuit the grid current meter can be calibrated to give direct readings of the amount of external resistance loading the resonant circuit

The measurement of the capacitance variation of the solution accompanying concentration changes makes it possible to achieve a still higher sensitivity to minute changes in concentration Use is made of the phase shift of the variable oscillator caused by the change in capacitance across its resonant circuit For this purpose a quartz crystal controlled reference oscillator operating at a frequency of 1085 kc/s is employed This supplies a fixed comparison signal to a phase-difference detector and also, through a very loose coupling a synchronizing signal to the frequency variable oscillator which is adjusted so as to operate at exactly the same frequency as the reference oscillator If the tuning capacitance of the resonant circuit of the variable oscillator is varied by only a small amount, say a few parts per 100,000 of the total capacitance (which includes that of the probe and solution) the synchronization will still be effective and the frequency remain unaltered but the sinusoidal oscillation will be advanced or delayed in time with respect to the oscillation of the reference, as has been indicated by the waveforms in Fig 1 This phase shift is measured by the phase detector, the meter of which gives readings proportional to the capacitance variation ΔC The circuit thus utilizes the well known and often harmful effect of 'pulling' between two oscillators, the proper operation requiring complete electro static shielding of each oscillator and careful control of the synchronizing signal

Except for solutions of low conductivity it is necessary to provide suitable means for limiting the absorption of energy by the electrolyte so as to ensure stable oscillations over a wide range of conductivities This has been achieved by inserting in series with one of the electrodes a capacitor, the optimum value of which depends on the dimensions, shape and spacing of the electrodes The series capacitor located close to the electrode is thus an integral part of the probe formed by the electrodes and has a great influence on its characteristics (*cf.* below) Depending on the conductivity of the particular solution into which the probe is inserted, a change in concentration affects the reading of the ΔI_g meter, or of the ΔC meter, or of both At increasingly higher conductivities the electrodes of the probe become practically short-circuited by the conductance of the solution, and the sensitivity curve of the reflectometer falls off

The circuit diagram of the reflectometer is essentially the same as that of the capacitance meter, constructional details of which have been given elsewhere (Haapanen 1962) For the present purpose a grid current meter was added and provisions were made for displaying the quantities ΔI_g and ΔC on a cathode ray oscilloscope, thus allowing the recording of rapid concentration variations The response of the reflectometer to a stepwise change in conductivity is such as to yield a rise time of 1 msec or less

2 *Measurements of changes in electrolytic conductance*

Capacitance variations as index of conductivity changes In order to obtain an idea of the general characteristics of the method and of its sensitivity as compared to that of conventional low-frequency determinations of conductivity, the main series of measurements was made employing as a probe for the reflectometer an arbitrarily chosen commercial conductivity cell (Philips type PR 9510) This probe consists of two platinum electrodes of about 8 × 8 mm covered with platinum black, placed in parallel 8 mm apart, and its cell constant when used for ordinary conductivity measurements is 0 77 Data will also be given on the properties of probes with electrodes of smaller area

The combined characteristics of the probe and solution were determined in the following way The probe connected to the reflectometer was lowered into a vessel containing distilled water together with another conductivity cell of the same type connected to an audio-frequency impedance bridge (General Radio Z Y bridge model 1603 A) by means of which the conductivity of the solution was measured in the conventional way The conductance of the distilled water was increased in steps by adding small amounts of dilute KCl solution Care was taken to insert the electrodes completely into the solution so as to avoid effects due to variations of liquid level The liquid was continuously stirred and allowed to reach concentration equilibrium before readings were taken

A difference method was used for measuring the capacitance changes of the probe solution For this purpose two variable standard capacitors of the coaxial type (*cf* Attree 1952) were used the first of a range of 1 pF allowing readings of 1 femtofarad to be taken the second of a range of 10 pF with a reading accuracy of 10 femtofarads (1 femtofarad = 10^{-15} F) The standard capacitor was connected in parallel to the input of the reflectometer and changes in probe capacitance as "seen" by the meter through the series-connected fixed limiting capacitor were balanced by varying the standard using the reflectometer as a null detector The determination of the relation between the capacitance of the probe solution and the conductivity of the solution by the difference method utilizes the high sensitivity of the reflectometer to capacitance changes but in other respects the characteristics of the reflectometer are not involved This means that the capacitance variation thus found can be expected to apply also when the same probe is used with other capacitance measuring instruments at the same frequency of 1085 kc/s

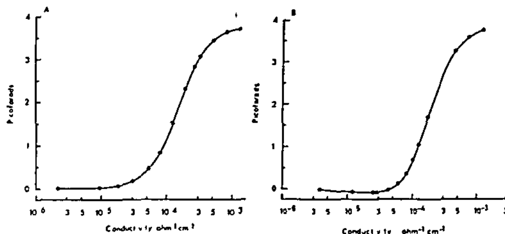


Fig 2 Relation between capacitance change of probe solution and conductivity of electrolyte A, under normal conditions B resonant circuit inferior to that in A See text

In Fig 2 A is shown the capacitance variation of probe/electrolyte obtained when the conductivity of the solution was increased The S-shaped curve shows that the major part of the capacitance change occurs between conductivities of 10^{-4} and 10^{-3} ohm^{-1} cm^{-1} and that the total change within this range is about 3 7 pF Similar measurements were made using series capacitors of 5 5 pF and 1 7 pF

A somewhat puzzling effect was observed when the measurement illustrated in Fig 2 A was

an increment in conductivity resulting in a decrement in probe capacitance This effect has been mentioned in an earlier investigation (Haapanen and Skoglund 1963) A secondary effect of inserting the padding capacitor is a decrease in the impedance of the resonant circuit This implies that there should be a critical value of loading on the resonant circuit at which the sign reversal as recorded by the reflectometer appears By loading the resonant circuit with carbon resistors of different values it could actually be demonstrated that for resistors greater than approximately 0 75 megohms the capacitance changes of the probe in dilute solutions were always recorded as being positive for increments in conductivity whereas for a loading resistor less than about 0 5 megohms an initial negative capacitance change was recorded For intermediate loadings, between 0 5 and 0 75 megohms the sensitivity to conductivity changes in dilute solutions below about 2×10^{-5} ohm^{-1} cm^{-1} was reduced The measurements show that in order to avoid the initial negative capacitance change the impedance of the resonant circuit of the reflectometer should be kept high This can be achieved by the use of low loss high quality components for the resonant

were therefore considered necessary

The total capacitance variations illustrated in Fig 2 are so large as to occupy several hundred full scales of the ΔC meter In order to determine the sensitivity of the probe to small increments in conductivity, the capacitance changes within small conductivity intervals were averaged, making use of the values from the measure-

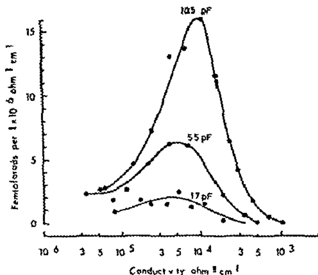


Fig 3 Change in capacitance for a change in conductivity of 1×10^{-6} ohm^{-1} cm^{-1} at different conductivities of the test solution Three values of series capacitor

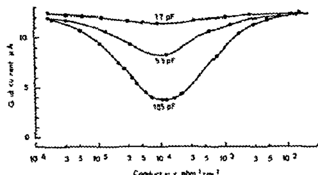


Fig 4 Grid current versus conductivity Same three values of series capacitor as in Fig 3

ments above The curves in Fig 3, representing the rate of capacitance change at different conductivities, give the sensitivity in terms of femtofarads capacitance change of the probe/solution per 1×10^{-6} ohm^{-1} cm^{-1} change in conductivity It is seen that the sensitivity is determined by the value of the limiting series capacitor and that a sensitivity maximum is attained at conductivities around 10^{-4} ohm^{-1} cm^{-1} An increased value of series capacitor shifts the peak of sensitivity into a higher conductivity range However, even for the largest value of capacitor used, *i e* 105 pF, the sensitivity curve is seen to fall off to virtually zero at conductivities higher than 10^{-3} ohm^{-1} cm^{-1}

Grid current as an index of loading The changes in the resistive component of the solution with changes in concentration are measured by means of the grid current Using the same probe and the same three values of coupling capacitors as before a measurement was made of the grid current as a function of the conductivity and the relation thus found is shown in Fig 4 As was to be expected the loading increases with the value of the coupling capacitor, a too large capacitor causing oscillat

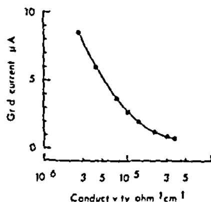


Fig 5 Grid current versus conductivity when series capacitor is omitted

be extinguished altogether at higher values of conductivity A comparison between Fig 3 and 4 shows that the grid current characteristics cover a wider range of conductivities, i e approximately four decades, as compared to about two decades for the capacitance changes It can further be seen that also for the grid current characteristics an increased value of the coupling capacitor shifts the peak of loading corresponding to maximum power absorption into a higher conductivity range For each combination of probe and series capacitor an individual calibration can be made, relating the grid current to the conductivity, making absolute measurements possible over a range of four decades Except for the minimum value of grid current, the same reading will be obtained for two different values of conductivity, making it necessary to pay due attention to the sign of the grid current change caused by a small increment in conductivity

A measurement was made on the loading effect of the probe when no series capacitor was used this arrangement resulting in a grid current curve of maximum possible slope shown in Fig 5 Even the conductivity of a solution of 2.5×10^{-6} ohm^{-1} cm^{-1} places a considerable load on the reflectometer, and increasing the conductivity to about 3.8×10^{-5} ohm^{-1} cm^{-1} causes the reflectometer to go out of oscillation This happens at a critical load corresponding to about 20,000 ohms

Overall sensitivity of IC recording It should be observed that the absolute sensitivity of *the reflectometer to incremental capacitance changes at its input terminals* is dependent on the amount of loading reflected from the probe For instance, the full linear range of the reflectometer corresponding to an output voltage variation of 90 volts at the plate of the phase discriminator is covered by a capacitance change of 8 femtofarads when the probe is in air When the probe is lowered into a solution of conductivity 2.5×10^{-6} ohm^{-1} cm^{-1} 11 femtofarads are required for the same output voltage For further increase of the conductivity to 10^{-4} ohm^{-1} cm^{-1} a capacitance variation of 30 femtofarads is needed Thus, for example, a change in conductivity of 1 % at 10^{-4} *ohm⁻¹ cm⁻¹ results in an output signal of about 50 volts* Paying due attention to the zero stability of the capacitance meter this means that conductivity changes of 10^{-7} ohm^{-1} cm^{-1} can be measured with reasonable accuracy and transient

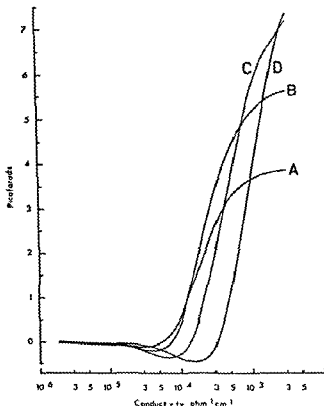


Fig 6 Capacitance change in relation to conductivity for four different probes, interelectrode spacing 8 mm in all cases Probe A, square electrodes 64 mm², B circular, 7 mm², C square 1 mm², D, circular, 0 3 mm² Same arrangement as in Fig 2 B See text

changes one order of magnitude less can be detected From a comparison between Fig 3 and 4 it appears that the maximum rate of capacitance change of the probe occurring at a conductivity of about 10^{-4} ohm^{-1} cm^{-1} roughly coincides with maximum load reflected from the probe Increasing the conductivity beyond 10^{-4} ohm^{-1} cm^{-1} or decreasing it below this value causes the resistive loading to decrease and consequently, the sensitivity of the reflectometer proper to increase The net effect is a tendency of the overall sensitivity of the recording system probe and reflectometer to be levelled out over a wider range of conductivities

When recording conductivity changes at high sensitivity *i e* when using a high value of series capacitor to the probe or high subsequent amplification, careful control of the temperature of the solution is necessary because of the high temperature coefficient of an electrolyte The change in conductivity with temperature is around 2 % per degree centigrade for most electrolytes Especially at the high relative sensitivity of the ΔC indication instability may result An example of the temperature rise that can be caused by the high frequency current flowing through a probe of small dimensions is given elsewhere (Haapanen and Skoglund 1967)

It is obvious from the measurements referred to that the choice of either ΔC or H_z indication makes it possible to achieve a high sensitivity over a wide range of conductivities As compared to conventional low-frequency methods of measuring

conductivity it appears that the sensitivity of the ΔI_g indication is similar to or better than that of most commercial conductivity bridges The sensitivity of the ΔC indication can be characterized as being very high, a limit to the smallest detectable conductivity change apparently being set by the attainable stability of the temperature

Effects of variation of electrode areas A few experiments were performed in order to study the effects of changing the electrode areas Four probes were investigated, all with the same spacing of 8 mm between the electrodes, the areas of which however varied from 64 mm² to 0 3 mm² In addition to a probe coupling capacitor of 10 5 pF, a padding capacitor of 500 pF was placed in series with the inductor which resulted in an initial negative capacitance change (*cf* Fig 2 B) As appears from Fig 6 a reduction of the electrode area augments the initial negative capacitance change and causes the shift to positive capacitance changes to appear at a higher value of conductivity A probe of small area is further seen to give a greater total capacitance swing than a large area probe, the limit being a capacitance swing approaching the value of the coupling capacitor It appears that for measurements within a specific conductivity range there is an optimum configuration yielding maximum sensitivity Thus for instance, in the conductivity range between 5×10^{-5} and 1×10^{-3} ohm^{-1} cm^{-1} the probe of characteristic B in Fig 6 is superior to that of characteristic A Similarly, at conductivities higher than 5×10^{-4} ohm^{-1} cm^{-1} the small area probe of characteristic D has the steepest slope However, in spite of the high ratio between the largest and the smallest area of the four probes, approximately 200 1, the resulting spacing of the characteristics by about one decade of conductivity is surprisingly small

For measurements requiring probes of special characteristics individual measurements have to be made One example of such a specially designed probe measuring conductivity changes in a small volume of solution and possessing in addition a shielding effect in certain directions has been described in a subsequent paper (Haapanen and Skoglund 1967)

3 *Interpretation of the results in terms of equivalent electrical circuits*

The basic theories of high frequency titration methods, especially with respect to the properties of solution and titration vessels and the types of response obtained from various instruments have been discussed by Blaedel *et al* (1952), Hall (1952 a) and by Reilley and McCurdy (1953) It was shown that titrimeters responding primarily to a change in capacitance or frequency had an S shaped response versus concentration curve, and similar curves were obtained also for the capacitance changes as measured by means of the probes used in this work (Fig 2 and 6) On the other hand, instruments based on measurement of grid or plate current or voltage of the high frequency oscillator tube were shown to exhibit a hump shaped response curve like the grid current characteristics in Fig 4

In order to explain and predict the response of an instrument based on the measurement of high frequency electrolytic absorption it is convenient to use an electrical

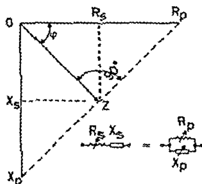


Fig 7 Vector diagram showing transformation of series components into equivalent parallel components. See text

analogue to the solution When used to replace the solution, this equivalent circuit, necessarily consisting of a combination of resistance and capacitance, should have virtually the same effect on the instrument as this solution While it is easy to think of the electrolyte as a concentration dependent resistor, the visualization of the capacitance associated with the conduction of electric current is not so straightforward In part, the difficulty is due to the influence of frequency on the capacitive component of the solution, the value of the reactance of a capacitor is dependent on the frequency, the value of a resistor is not The early observation (Forman and Crisp 1946) that a change in concentration caused a variation in the capacitive component in spite of the frequency remaining practically constant suggested that the dielectric constant of the solution either was concentration dependent or underwent an 'anomalous dispersion"

In the papers by Blaedel *et al* (1952) and by Hall (1952 a) the same equivalent circuit was suggested for the solution proper, *viz* a resistor and a capacitor in parallel, the values of both components being considered to vary with the concentration, thus implying a change in the dielectric constant However, it was suggested by Hall that the variation in capacitance might also be accounted for by assuming the equivalent circuit to consist of a varying resistor in series with a capacitor of fixed value For the purpose of illustrating how a change only in the series resistance actually can affect the net capacitance of such a circuit, the application of a graphical method of analysis is convenient

It should be observed that the conception of the electrolyte as a series circuit of resistance and capacitance cannot be verified by direct measurement, the terminals of these components being inaccessible An instrument such as the reflectometer therefore actually measures the impedance of the solution in terms of parallel components To check the validity of the series circuit as representing the solution an equivalent parallel circuit must therefore be found possessing the same characteristics

According to electrical network theory any impedance Z formed by a resistance R_s and a reactance X_s in series may also be formed by another resistance R_p and another reactance X_p in parallel A transformation from one form to the other can be made either by using a set of well known equations giving the relationships between series and parallel equivalents or by means of a geometric method The latter shown in Fig 7 is rapid and capable of providing general solutions

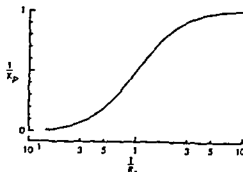


Fig 8 Relation between $1/X_p$, corresponding to parallel capacitance, and $1/R_s$ corresponding to series conductance Curve constructed according to the method shown in Fig 7, assuming constant series capacitive reactance X_s See text Cf Fig 2 A

at right angles to Z at its end The diagram is drawn in a proportion such as to make both parallel components assume a value twice that of the equivalent series components It is to be observed that the capacitive reactance X_p represents a capacitor only half as large as that corresponding to X_s (The capacitive reactance X_s is numerically equal to $1/2\pi fC$, where f = frequency and C = capacitance)

The effect of varying the series resistance R_s while keeping the capacitive series reactance X_s at a fixed value can now be studied For this purpose, R_s is given various values and the line R_p—X_p is constructed for each value of R_s The end of the vector Z will thus move along the line X_s—Z and its extension to the right With respect to the parallel components it will be found that not only the value of R_p but also that of X_p will vary This result has been indicated in the small circuit diagrams in Fig 7

If R_s is taken to represent the series resistance of an electrolyte, the corresponding conductance of which is $1/R_s$, and using the method described, a curve can be constructed giving the relation between the conductance $1/R_s$ and $1/X_p$ The variable $1/X_p$ represents the value of the capacitance C which corresponds to the capacitive reactance X_p By assigning R_s values between 0 1 and 10 and plotting $1/R_s$ on a logarithmic scale, the curve shown in Fig 8 is obtained Comparing this curve with that given in Fig 2 A, showing the relation between the capacitance of a probe and conductivity of the solution, both curves show the S-shaped capacitance variation to be completed within about two decades of conductivity From the similarity of the two curves the conclusion can be drawn that the series equivalent circuit is a useful approximation, simulating the behavior of the electrolyte and showing that the capacitance changes actually measured can be explained on the basis of a change in resistance only

It is thus not necessary to assume a change in the dielectric constant of the solution with conductivity The question whether the change in capacitance caused by a change in conductivity is due to a real or an apparent change in the dielectric constant of the solution has for a long time been a matter of controversy because of

the difficulties involved in the measurement of the dielectric constant of electrolytes On the basis of recent measurements it seems likely that for dilute solutions the change in dielectric constant is very small if at all existent (*cf* Smyth 1955) This supports the concept of the equivalent circuit of the electrolyte as a series connection of a varying resistance and a fixed capacitance

In the geometric analysis described above no attention has been paid to the effect of the limiting capacitor placed in series with the electrodes of the probe However, when the solution is represented by the series equivalent circuit account can be taken of the effect of this capacitor simply by adding the corresponding value of capacitive reactance to the value of X_s of the solution The limiting capacitor of the probe corresponds to the capacitance across the walls of a titration vessel, the conditions for which can thus be analyzed by the same method

The phenomenon of maximum absorption of energy appearing at a particular concentration of the solution can also be illustrated by this graphical method If the value of series resistance R_s is continuously decreased starting from high values (corresponding to dilute solutions) it will be seen that the parallel equivalent R_p will first decrease, pass a minimum and then increase again The condition for obtaining the minimum value of R_p, *i.e.* the maximum loading effect, appears from the vector diagram which shows that this happens when the series resistance R_s is made equal to the series reactance X_s, the phase angle of the impedance Z being 45° The influence of the series capacitor of the probe on the position of the peak of loading, which was illustrated in Fig 4, can now be explained Adding the capacitive reactance of the series capacitor of the probe to the reactance X_s of the electrolyte results in an increased total capacitive reactance In order to equal this value, the series resistance R_s of the solution must therefore also be increased, which is equivalent to a decreased conductivity It is thus obvious that the introduction of the series capacitor shifts the peak of loading into a range of lower conductivity, the lower the smaller the value of the series capacitor

The influence of the frequency on the value of concentration for maximum power absorption as predicted by the empirical relation given by Forman and Crisp (1946) can be accounted for by considering the dependence of the capacitive reactance on frequency (*cf* above) For instance, a tenfold increase in frequency results in a capacitive reactance one tenth of the original value, the value of capacitance remaining unaltered For the example given this means that the value of the series resistance R_s must be decreased to one tenth in order to equal the value of X_s (*cf* Fig 7) Thus maximum absorption would occur at a conductivity ten times the original one

For the purpose of visualizing the effect of varying the conductivity of the solution or of changing the parameters of the probe or of choosing another operating frequency the graphical analysis is thus a powerful tool By this method it has been possible to show that a series circuit of a varying resistance and a fixed value capacitance is a useful analogue of an electrolyte simulating several high frequency properties of the solution

This work has been supported by grants from the Swedish Medical Research Council, the foundation "Therese och Johan Anderssons minne" and the foundation "Gustaf och Tyra Svenssons minne"

References

ATTREE, V. H., A simple capacitance-meter using a crystal-oscillator *Electronic Engng* 1952 24 284—286

BLAEDEL, W. J. and H. V. MALMSTADT, High-frequency titrations A study of instruments *Anal Chem* 1950 22 734—742

BLAEDEL, W. J., H. V. MALMSTADT, D. L. PETITJEAN and W. K. ANDERSON, Theory of chemical analysis by high frequency methods *Anal Chem* 1952 24 1240—1244

BRADFORD, H. K., High-speed analysis of electronic circuits by geometry *Electronics Buyers' Guide* 1961 R 50—R 64

FORMAN, J. and D. J. CRISP, The radio-frequency absorption spectra of solutions of electrolytes *Trans Faraday Soc* 1946 42 A 186—194

HAAPANEN, L., A high-sensitivity capacitance meter *Electronic Engng* 1962 34 183—185

HAAPANEN, L. and C. R. SKOGLUND, Recording of net ionic flux during a single action potential in *Nitellopsis obtusa* *Acta physiol scand* 1963 59 297—298

[illegible]

HOLCOMB, W. G., C. E. ANAGNOSTOPOULOS and W. W. L. GLENN, A new method of determining tissue resistance by the reflectometer principle, for use with implanted RF pacemaker receivers, laboratory and clinical observations on cardiac, bladder and phrenic nerve pacemakers *Trans N. Y. Acad Sci* 1965 27 894—908

LAMB, D. E., F. S. MANNING and R. H. WILHELM, Measurement of concentration fluctuations with an electrical conductivity probe *A I Ch E Journal* 1960 6 682—685

REILLEY, C. N. and W. H. MCCURDY, JR., Principles of high-frequency titrimetry *Anal Chem* 1953 25 86—93

SMYTH, C. P., Dielectric behavior and structure *McGraw-Hill Book Co., Inc* New York 1955

Acta physiol. scand. 1967. 69. 51—68

From the Department of Physiology, Karolinska institutet, Stockholm, Sweden

Recording of the Ionic Efflux during Single Action Potentials in Nitellopsis Obtusa by Means of High-Frequency Reflectometry

By

L HAAPANEN and C R SKOGLUND

Received 29 June 1966

Abstract

HAAPANEN, L and C R SKOGLUND *Recording of the ionic efflux during single action potentials in Nitellopsis obtusa by means of high-frequency reflectometry* Acta physiol scand 1967 *69* 51—68

[illegible]

temporal relation between the ionic concentration change and the action potential, that the method is suitable for quantitative measurements is demonstrated by inkwriter records showing the stepwise increases in concentration caused by intermittent stimulation during hour long experiments Quantit t ve data on the increments in ionic concentration associated with a single impulse, and on the total increase resulting from a number of stimuli are presented and compared with chemical analyses showing the ions involved to be K and Cl Satisfactory correspondence was obtained between the values of the conductivity measurements tentatively expressed as KCl concentration and the chemical analysis The average values for the K efflux were 2×10^{9} to 4×10^{9} pmole/cm^{2} impulse preliminary studies showed the Cl efflux to be of approximately the same order

The quantitative data available about the ionic exchange associated with a single action potential in various types of excitable tissues represent average values derived from a great number of stimulations (*cf* Hodgkin 1964) Also our knowledge about the temporal relationship between the ionic fluxes and the electrical events obtained by fractionation of tracer effluxes is based on experiments with repeated stimulation (*cf* Sjostrand 1964) However, it would obviously be desirable to be able directly to record the ionic changes associated with each individual action potential in a series of activations, and any method of sufficient sensitivity and time resolving power for such a dynamic recording seems worth trying

We have previously reported a first series of experiments on internodal cells of *Nitellopsis obtusa* aimed at developing a method for direct recording of the ionic outflow during excitation (Haapanen and Skoglund 1963). Algal cells were chosen because of their slow activation processes and the concomitant large ionic exchange. Thus, as shown by Gaffey and Mullins (1958) by means of tracer technique, the efflux of K^+ during an action potential of *Chara globularis* is about ten thousand times larger than in squid nerve. The authors did not find any change in the resting Na^+ influx during excitation but could demonstrate an increase in the Cl^- efflux and suggested that the action potential in *Chara* is associated with an initial outflow of Cl^- followed by a later outflow of K^+. If the ionic mechanism is of the same type also in *Nitellopsis*, the total ionic exchange during excitation would then be reflected as an increase in ionic concentration of the extracellular fluid surrounding the alga. This can be recorded as a change in conductance, and in our preliminary paper a suitable method for measurements of such changes was reported.

With this method, the principles of which have been described by Haapanen (1967), changes in ionic concentrations are measured as variations in absorption of a high frequency field applied by a special probe immersed in the extracellular fluid. As will appear from the present paper, this method of high frequency reflectometry has sufficiently high sensitivity and time resolving power to permit a dynamic recording of the ionic efflux during a single action potential of an alga. The dynamic aspects will however not be dealt with in this paper, the primary aim of which is to establish that the method is suitable for quantitative recording of the ionic efflux during activation and also to show by conventional chemical methods that the ions involved in excitation of *Nitellopsis obtusa* are Cl^- and K^+.

The results obtained were briefly reported at the XII Scandinavian Physiological Congress (Haapanen and Skoglund 1966).

Methods

Material. The experiments were performed on cells of *Nitellopsis obtusa* collected in the autumns of 1964 and 1965 from the lake of Erken, located some 50 miles north of Stockholm. The plants from each collection were divided into two batches, one of which was stored in a solution consisting of 90 % distilled water and 10 % tap water. Cells from this batch were usually chosen for the main series of experiments using distilled water as the initial external medium. The other group of plants was stored in water taken from the lake and used for a minor series of experiments on algae immersed in lake water. The ionic constituents of this water are shown in Table I which also gives the ionic concentrations in the sap of the algae (*cf.* below).

The plants were kept under cool conditions in semidarkness. The tanks were inspected frequently and were rinsed and filled with new water as soon as they showed signs of contamination. After an

TABLE I. Ionic composition of the lake water of "Erken" and of the sap of *Nitellopsis obtusa* (mean values obtained from determinations on 10 algae)

Ion	K^+	Na^+	Ca^{++}	Mg^{++}	Cl^-	SO_4^{--}	HCO_3^-
Lake water mM	0.05	0.27	2.37	0.37	0.21	0.82	2.11
Sap mM	150	0.65	10		167		

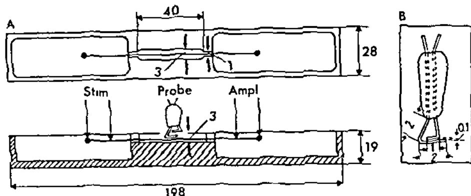


Fig 1 A, schematic drawing of perspex test chamber showing dimensions in mm and standard *arrangement for recording and stimulation*, *B*, *reflectometer probe*

by means of either Dow Corning Silicone Grease or acid free vaseline The dimensions of the

At the beginning of an experiment all compartments of *the test chamber were filled either with* distilled water obtained from an ion exchanger and of a conductivity about 0.5×10^{-6} to 1×10^{-6} ohm^{-1} cm^{-1}, or with lake water of the composition given above The increments in conductivity of the test solution accompanying action potentials in the plant cell or caused by spontaneous ionic leakage from the cell were detected by the conductivity *probe connected to the high frequency* reflectometer

The high frequency reflectometer used in these experiments

$= 10^{-14}$ F) obtained from appropriate capacitive transducers The present application of the instrument is based on the exposure of a sample volume of the test solution to the high-frequency field

high frequency oscillator has to deliver *to a load the more sensitive it becomes to* variations in this load (This effect is often referred to as reflection from the load towards the oscillator, hence the term reflectometer

pl

va

in

ec

sn

pedance In the following these two *methods of recording* conductivity changes will be referred to

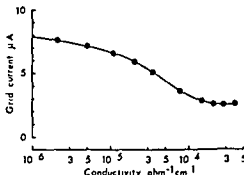


Fig 2 Relation between I_g values as indicated on the reflectometer scale and conductivity of test solution

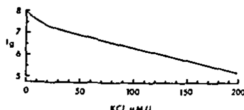


Fig 3 Relation between I_g values and KCl molarity

I_g and C recordings, respectively These variables were indicated on separate meters and could also be displayed on an oscilloscope or recorded on an inkwriter Further data about the working principles of the reflectometer are given in a separate paper (Haapanen 1967), and the present account will be concerned mainly with the characteristics of the instrument when used together with the particular probe described below

It was soon observed that the probes used in our preliminary investigation (Haapanen and Skoglund 1963) had certain disadvantages When these probes were placed directly against the cell wall the cells became exposed to a high frequency current which in prolonged experiments caused damage to the cell wall, resulting in ionic leakage at the site of the probe In addition, it appeared necessary to increase the selectivity of the probes and to restrict the recording of concentration changes to a well defined volume of fluid

Lamb Manning and Wilhelm (1960), describing an audio-frequency method of concentration measurement within a small volume of electrolyte, pointed out that it is hard to predict the modification of the potential fields occurring for inhomogeneous fluids in which spatial variations of concentration occur In our case further complications were encountered owing to the abrupt discontinuities in conductivity and dielectric constant at the interface between the test solution and the cell wall as well as at the walls of the test compartment Since the primary aim of the present in [illegible]

the probe shown in Fig 1 B It was made by melting glass powder (Corning no 7570 solder glass) around the legs of a L shaped piece of unplated wire consisting of 90 % platinum and 10 % iridium and of 0 4 mm diameter When the middle parts of its legs were well embedded in glass the wire was cut and bent as shown in the figure The inner and outer electrodes are 0 1 mm apart in the interspace the density of the high frequency field is maximal In series with the inner electrode is a fixed-value capacitor of 10 5 picofarads the function of which is to limit the maximum amount of power absorption from the reflectometer (*cf* Haapanen 1967) The outer electrode was usually placed in contact with the cell wall the inner electrode being then at a fixed distance of 0 5 mm from the alga surface The outer larger electrode was connected to the grounded terminal of the reflectometer, thus acting as a shield against the plant cell (for efficiency of shielding *cf* below) By placing the probe with the shaft of the grounded electrode in the direction of the approaching impulse a shielding effect was also obtained against the discontinuity of ion concentration resulting from activation of the alga segment proximal to the site of the probe The probe can be characterized as a hybrid between a normal conductivity cell and a purely capacitive transducer

The effect of the *resistive loading* reflected from the probe to the reflectometer appears from Fig 2 which shows the relation between the grid current I_g and the conductivity of the electrolyte Within a conductivity range from that of distilled water of 1×10^{-6} ohm⁻¹ cm⁻¹ and up to about 2×10^{-4} ohm⁻¹ cm⁻¹, increments in conductivity give decrements in grid current When continuing the measurements beyond 3×10^{-4} ohm⁻¹ cm⁻¹ the grid current could be shown to increase again The grid current is thus a good indicator of small changes in the conductivity of dilute solutions up

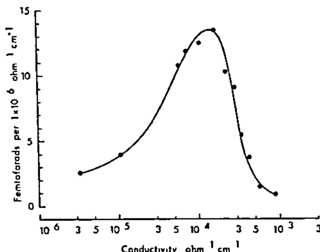


Fig 4 Change in capacitance of probe/solution for a change in conductivity of 1×10^{-6} ohm^{-1} cm^{-1} at different conductivities of the test solution

[illegible]

found to be the method of choice for the lake water experiments in view of its high sensitivity and in spite of the somewhat more complex interpretation of the results obtained

The gain and balance controls of the inkwriter amplifier were used to offset the zero and expand [illegible]

to the instrument real I_g values are obtained by multiplying by 1.5) the grid current recorder was fitted out with two more scales one showing conductivity and the other calibrated in molarity KCl solution (cf Fig 9) [illegible]

about 13.5 femtofarads This occurs at a conductivity of approximately 2×10^{-4} ohm^{-1} cm^{-1}, i.e. close to that of the lake water 2.4×10^{-4} ohm^{-1} cm^{-1}

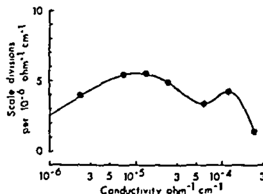


Fig. 5. Overall characteristics of probe and reflectometer for C recording. See text.

When studying conductivity changes by means of C recording it should be observed that there is an interaction between loading and the sensitivity of the reflectometer to a given amount of capacitance change (*cf.* Haapanen 1967). The overall sensitivity expressed in terms of scale divisions of the C recorder for a conductivity change of 1×10^{-6} ohm^{-1} cm^{-1} is shown by the curve in Fig. 5. Although the initial conductivity of the lake water is about two orders of magnitude higher than that of distilled water, the ΔC sensitivity in lake water is approximately the same as the ΔI_g sensitivity in dilute solutions.

When testing the shielding effect of the probe with respect to the C recording as described above, it was found that in lake water and when probe and cell were less than 0.2 mm apart there was a slight effect on the ΔC reading showing a small amount of residual coupling to exist. Thus, it cannot be excluded that a small component in the C recordings performed with lake water as test solution may be of intracellular origin or may be caused by a probe extension effect due to the high conductivity of the cell sap. These effects diminish with increasing concentration, and it is thus possible that during the actual recording conditions in lake water experiments the influence of residual coupling may be reduced to a second-order effect. In more dilute solutions the effects of residual coupling become more pronounced, which has to be taken into account when applying C recording in experiments on algae in distilled water.

With regard to the dynamic characteristics of the reflectometer, the rise time in response to a step function is of the order of 1 msec. This response time is likely to be shortened when the reflectometer is loaded by a solution.

A source of error that may become serious when the concentration of an electrolyte is determined by means of its electrical conductivity is the influence of temperature changes. For most electrolytes the temperature coefficient of the conductivity is of the order of 2 per cent per degree centigrade. During recordings made with the reflectometer care was taken to minimize temperature variations in the laboratory, most experiments being performed at about 22 °C. However, no special attempts were made to stabilize the temperature of the test chamber, and hence it was rather surprising that the baseline stability of the ΔI_g indication during long test runs on standard solutions was actually quite satisfactory. A similar stability could not be attained for the C recording in lake water, most likely due to the higher relative sensitivity of the latter mode of recording.

It could be anticipated that the power absorption of the solution should result in a local temperature rise in the vicinity of the probe. To check this, a small temperature transducer was immersed in the test solution close to the probe placed in its normal recording position but in the absence of an alga. In a KCl solution of 100 μM the temperature rise proved to be about 0.8 °C, whereas in lake water, in which the power absorption is close to maximum, the rise amounted to 1.5 °C. On the average, this temperature rise, if uncorrected as in these experiments, should thus result in a concentration reading about 2 % too high. Considering the overall errors in the measurements of the concentration of a KCl solution or the conductivity of an unknown solution, the accuracy of the method is estimated to be about $\pm 0.5 \times 10^{-6}$ ohm^{-1} cm^{-1} within the range of conductivities of interest in this study. These figures refer to the I_g recording at the gain used in these experiments.

Recording and stimulating equipment. Action potentials were recorded by means of a Grass model P6 preamplifier equipped with a symmetrical low-pass L-C filter (attenuation 50 dB at 1085 kc/s) at the input in order to prevent blocking of the amplifier by the high frequency carrier from the reflectometer. The action potentials were displayed on one channel of a Tektronix 502 two-beam oscilloscope and/or on one of the two channels of a Siemens inkwriter (time constant 1 sec), the other channel being used for either the I_g or the C recording. An Ampex FR 1300 tape recorder made possible a simultaneous recording of the action potential, ΔI_g and ΔC for subsequent playback

and analysis The stimulator was a Grass S4 with isolation unit, and Ag-AgCl electrodes were

or plastic

Determinations of the concentrations of K^+ and Na^+ (in some cases also of Ca^{2+}) in the samples were made by means of an Eppendorf flame photometer, later replaced by a Beckman B spectrophotometer with flame attachment

solution The measurements were made under a low power microscope so as not to cause damage to the electrode surface

A matched pair of the chlorinated electrodes was connected to the cathode follower input of a Grass model P6 preamplifier adjusted for minimal grid current (about 10^{-13} A), and the potential difference developing between the electrodes was recorded on a Hewlett Packard model 425 A microvoltmeter connected to the output terminals of the P6 preamplifier With both electrodes in the same chloride solution a maximal potential difference of 1 mV was allowed The electrodes

the test solution the sample was transferred to one of the vessels and compared to a standard KCl solution in the other, the procedure otherwise being the same Since the Cl^- concentration value to be expected was roughly known from the corresponding I_g recording, standard solutions of concentrations within a narrow range above or below the actual value could usually be chosen, which facilitated the measurements As applied in the present investigation the method cannot be claimed to have an accuracy better than ± 10 %

Analysis of sap A number of sap samples were analyzed for concentrations of K^+, Na^+, Ca^{2+} and

flame photometry and for Cl^- by the potentiometric method In another series of measurements determinations of the chloride concentrations of undiluted 10 μl sap samples were made using the Beckman model 150 Microtitrator For results see Table I

Results

Algae in distilled water

In agreement with earlier findings on other types of algae (*e g* Blinks 1955), *Nitellopsis obtusa* was found to maintain its excitability for a considerable time when immersed in distilled water, and this property was utilized in the series of experiments to be described in this section That the activation processes studied are not significantly disturbed under these conditions has been verified by comparative experiments performed on algae in their natural lake water milieu (*cf* next section) Starting from distilled water as test solution makes it possible to use the I_g recording as an index of the extracellular concentration changes, and this offers certain advantages from an analytical point of view (*cf* Methods)



Fig. 6. Changes in ionic concentration as recorded by the probe in normal position (A), and 2 mm farther away from the alga (B), in relation to the action potentials (lower beams). Vertical bar = 1×10^{-6} ohm^{-1} cm^{-1}. Time bar 10 sec.

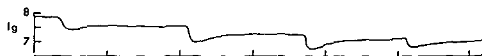


Fig. 7. Complete time courses of ionic concentration changes resulting from repeated stimulations (at artefacts) of an alga. Tracing of inkwriter record. Time marking every 5 min.

In this main series of experiments all three compartments of the perspex chamber were thus filled with distilled water. Before starting the experiment the purity of the water surrounding the alga inside the sealed test compartment was checked by reading off the I_g value on the reflectometer. Very often, contamination of the test solution made it necessary to flush the test compartment and refill it with new water. The conductivity of the test solution was always monitored on the inkwriter for a certain period of time, usually 10—15 min, before proceeding with the experiments. Only when the I_g recording during this time had shown that the conductivity was maintained at the value of distilled water, or was stabilized at a slightly higher value, was the actual experiment started by delivering the first stimulus. Cells which due to an abnormally high resting efflux did not attain a constant I_g level within the stabilizing period were discarded.

The present paper does not aim at a close analysis of the temporal relationship between the action potential and the accompanying ionic efflux. As an illustration, however, some typical records obtained by simultaneous display on the oscilloscope of the action potential and the conductivity changes as indicated by the I_g recording will be shown. In Fig. 6 A the decrease in I_g level, i.e. the increase in conductivity of the extracellular fluid as recorded with the probe in standard position, is seen to begin soon after the peak of the action potential. The conductivity change reaches maximum about 25 seconds later, in this record only the first sign of the subsequent characteristic return to a new plateau (cf. Fig. 7) is visible. In record B the probe was

Fig. 8. Stepwise increases in ionic concentration resulting from 16 consecutive stimulations of an alga at 7-minute intervals. Tracing of sequence of continuous record to be read from above downwards. Time marking every 5 min.

moved about 2 mm farther away from the alga surface, resulting in a significantly longer latency of the recorded conductivity change, as was to be expected from the increased diffusion distance.

The full time course of the conductivity change associated with the impulse is illustrated by the inkwriter record in Fig. 7. There is a successive return to a new steady level after each impulse, in this experiment attained about 4 minutes after the start of the change; this is apparently the time needed for the diffusion process within the test compartment to reach equilibrium.

The difference between a new plateau and the previous one represents the increment in conductivity caused by one action potential. These steps of increased ionic concentration may vary from one cell to another and also from one impulse to the next in a series of activations of one and the same alga. The conductivity changes in Fig. 7 are larger than the average ones which are recorded in Fig. 8 at the same amplification. This cell was stimulated 16 times at intervals of about 7 min and the grid current is seen to decrease in discrete steps from the initial I_g value of 7.9 to the final value of 6.0, which corresponds to an increase in conductivity from 1.1×10^{-5} to 2.0×10^{-5} ohm^{-1} cm^{-1} (*cf.* Fig. 2).

As already mentioned, the ions that in the first place can be assumed to account for this conductivity change are K^+ and Cl^-. At an early stage of the investigation we therefore decided tentatively to express the concentration change in KCl molarity, thereby also assuming an equal outflow of both ions, and then to submit the validity of these assumptions to a test by chemical analyses of the extracellular fluid.

The total conductivity change in the experiment in Fig. 7 corresponds to an increase in KCl concentration from 3 μM to 130 μM (*cf.* Fig. 3). The consecutive in-

TABLE II. Increment in ionic concentration of test solution expressed in μM KCl for each of the consecutive action potentials induced by repeated stimulation at 7 minute intervals

Action potential no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Sample volume μl
Exp. 32	2	5	7	12	8	8	6	9	9	9	8	9	9	8	9	9	195
Exp. 34	4	5	11	13	13	10	19	14	11	13							262
Exp. 13	15	15	13	12	13	12	12	11	12	7	10	11	6	8	8	7	—
Exp. 39	31	35	25	22	15	10	3										230

TABLE III Comparison of the values of the total ionic concentration expressed in µM KCl obtained by HI reflectometry, with those of the K^+ concentration obtained by flame photometry, in the test solutions after a varying number of action potentials See text

1	2	3	4	5	6	7
			Flame	Reflectometry		Flame
Exp no	Sample volume µl	Number of impulses	K^+ µM	KCl µM	K^+ 10^3 pmole/cm² impulse	K^+ (total) 10^3 pmole/cm² impulse
30 a	220	1	16	7–4	1 6	4 2
31 b	280	4	50	46–3	3 9	4 6
24 a	220	4	65	38–6	2 2	4 5
24 b	270	5	40	37–7	2 1	2 7
22 b	200	6	58	51–10	1 8	2 5
25	190	7	68	68–4	2 2	2 4
31 c	206	8	27	23–3	0 7	0 9
29	265	15	79	101 4	2 3	1 8
21 b	220	15	144	149–7	2 9	2 7
38 b	210	17	80	130–7	1 9	1 3
				Mean values	2 2	2 7

crements in concentration for each single impulse in this record are given in Table II (exp 32) As can be seen in the table, the increments become successively larger in the beginning of the series, and this trend is typical of most experiments, as exemplified also by exp 34 In the later parts of the series the steps are strikingly constant in exp 32, whereas there are somewhat larger variations in exp 34 Experiments 13 and 39 represent the less common finding, *i e* the increments in concentration become successively smaller The very rapid decrease in ionic efflux in exp 39 is probably a sign of an abnormal condition of the alga which in fact became inexcitable after the 7th stimulus No attempts were made in the present study to find out the causes of these variations in ionic efflux

Table III gives typical examples of experiments comprising different numbers of stimuli From column 5, showing the KCl concentrations of the test solution at the end and beginning of the experiment (first and second figures respectively) it can be seen how the concentration increases with the number of action potentials but it is also evident that there are individual variations in efflux between different algae These appear from column 6 which shows the average K^+ efflux per cm² impulse calculated on the assumption (verified below) that K^+ is the dominant cation These values are calculated on an average diameter value of 0 6 mm, but in reality the diameters were observed to vary between 0 5 and 0 6 mm However, the source of error thus introduced cannot explain the large variations in efflux between different algae These data will be further discussed below in connection with the results of the chemical analysis

Identification of ions involved In one series of experiments the test solution was collected for chemical analysis By means of flame photometry it was tested whether K^+ and Na^+ and if the sample volume permitted, also Ca^{2+}, were present In samples from algae showing no detectable leakage in stimulus free periods, Ca^{2+} was never found in any significant amounts and Na^+ was present, if at all, only in minimal concentrations corresponding to the normal resting efflux during the experimental period (*cf* MacRobbie and Dainty 1958) As was to be expected, K^+ was the only cation present in amounts large enough to account for the conductance change

The values in column 4, Table III, show the K^+ concentrations in the test solutions at the end of the experiments as determined by flame photometry When comparing these values with those of the final KCl concentration based on the I_g values (first figures in column 5) it is necessary to have in mind the various sources of error involved Thus, it is rather surprising to find such a good correspondence between the two values as in experiments 31 b, 24 b 25, 31 c and 21 b, the errors must in fact have cancelled out when the figures are identical, as in exp 25 A difference such as those exemplified by expts 30 a and 24 a, in which the values obtained by the reflectometric method are significantly lower than those by flame photometry, can be explained by contamination of the test solution after the last recording of the I_g value during the experiment This may happen during the collecting of the sample if the alga is damaged so that leakage occurs, or during the later handling of the sample The discrepancy in exp 30 a is exceptionally large which is not surprising considering the increased influence of various sources of error at the low ionic concentration resulting from a single impulse Thus for instance, these small increments in ionic concentration are in the vicinity of the resolving power of the flame photometer used

Expts 29 and 38 b illustrate cases in which the K^+ value obtained by flame photometry is too low to account for the I_g value Since no significant amounts of any other cation could be detected such a discrepancy is most likely due to methodological errors Thus contamination of the sample by less concentrated fluid from the outer compartment when sucking up the sample from the test compartment can easily occur if the sealing between the compartments is damaged during the procedure Such errors can be detected by determining the I_g value also after the transfer of the sample to the test tube used for chemical analysis

On the whole considering the sources of error the values obtained for the K^+ concentration by chemical analysis correspond sufficiently well to those based on the I_g values to permit the conclusion that K^+ is the dominant cation accounting for the recorded conductance change It should thus be justified to calculate the values of K^+ efflux per cm^2 impulse on the basis of the difference in KCl concentration at the beginning and end of an experiment as shown in column 5 Table III The figures thus obtained (column 6) illustrate that there are individual variations from a minimum of 0 7 to a maximum of 3 9 the average value being 2 2 $\times$ 10^3 pmole per cm^2 impulse for this series of algae

For comparison the values for the K^+ efflux per cm^2 impulse calculated on the

TABLE IV. Comparison of the values of the total ionic concentration expressed in µM KCl obtained by HF reflectometry, and the values of Na⁺, K⁺ and Cl⁻ concentrations obtained by chemical analysis in the test solutions after a varying number of action potentials. The experiments are arranged according to the increasing concentrations of Na⁺ found in the test solution as a result of abnormally large resting effluxes

1	2	3	4	5	6	7	8
			Chemical determinations µM				
Exp. no.	Number of impulses	Reflectometry KCl µM	$K^+ + Na^+$	Na^+	K^+	Cl^- corrected	Cl^- observed
15	7	100	89	7	82	51	58
18	10	135	108	8	100	112	120
17	10	140	105	17	88	110	127
4	4	75	63	19	44	23	42
5	8	125	111	24	87	76	100
7	10	135	151	34	116	106	140
1	9	200	162	56	108	94	150

final K⁺ concentrations obtained by flame photometry are given in column 7. In this case the mean value is 2.7, however, the values include an error due to the unknown amount of K⁺ present in the test solution at the beginning of the experiment. If one assumes the same relation between the initial and final K⁺ concentration as shown by the figures for the corresponding KCl concentrations in column 5 and corrects the chemically determined values accordingly, this would result in an average value for the K⁺ efflux of 2.1 as compared to 2.2 for the I_g based values.

For reasons given in the introduction, Cl⁻ could be expected to be the anion involved in the excitation process, and in some of the experiments in this early series an activation analysis of the test solution was therefore performed. Unfortunately, the high background activity of Cl⁻ in the sample container did not permit any conclusion to be drawn about the actual concentration of Cl⁻, it could only be inferred that there was an increase of Cl⁻ concentration in the extracellular fluid with increasing number of impulses.

When a suitable modification of the potentiometric method for quantitative Cl⁻ determinations had been worked out, only algae from the collection period of 1965 were available. Unfortunately, the analysis was complicated by the fact that these algae exhibited a larger than normal resting efflux of both Na⁺ and Cl⁻, as evidenced by chemical determinations of test solutions from experiments on algae without stimulation. On the basis of a series of experiments on some 20 algae, some typical results of which are presented in Table IV, it was however possible to get an estimation of the Cl⁻ outflow associated with activation.

A comparison of column 6 in Table IV with column 4 in Table III shows that the values of the final K⁺ concentration after a given number of impulses are of the

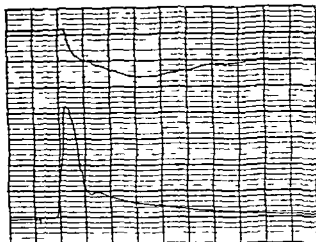


Fig 9 Alga in lake water Change in ionic concentration (upper curve) in relation to action potential (lower curve) Between vertical lines 25 sec

same order, and this indicates that in the two series of algae the ionic processes during activation are comparable in spite of the abnormal resting effluxes of Na^+ in the 1965 series It also appears that the sums of the K^+ and Na^+ concentrations (column 4, Table IV) agree fairly well with the total concentrations based on the I_g values given in column 3 Since these are expressed in KCl molarity certain discrepancies are in fact to be expected owing to the lower value of equivalent conductance of the Na^+ ions

In the majority of the experiments (18, 17, 5, 7 and 1) the values obtained for the Cl^- concentration (column 8) show sufficiently good correspondence with the KCl concentration based on the I_g value to allow the conclusion that Cl^- is the dominant anion present In experiments 15 and 4 the Cl^- values observed are too low to account for the observed conductivity changes, which might indicate the presence of other an- or cations However, the larger methodological errors at the low final concentrations in these experiments also have to be taken into account

Since, as mentioned above, the increased resting outflow of Na^+ occurring in these algae was accompanied by *an increased resting outflow of Cl^-*, a closer estimation of the Cl^- efflux associated with the impulse discharge is not possible Assuming a resting outflow of Cl^- matching that of Na^+, for which support was obtained in the experiments without stimulation, the Cl^- efflux during activation can be estimated by reducing the total Cl^- value by the Na^+ value The figures thus obtained (column 7, Table IV) show variations below and above the corresponding K^+ values However, except for expts 15 and 4 the differences are small, suggesting a release of Cl^- in approximately the same quantities as K^+

Algae in lake water

A minor series of explorative experiments on algae in their natural chemical milieu was performed to find out how the ionic processes might differ from those in distilled water At conductivities such as that of the lake water, 2.4×10^{-4} ohm^{-1} cm^{-1}, only

TABLE V *Comparative experiments on algae in lake water Increment in K concentration as determined by flame photometry in test solutions after a varying number of action potentials*

1	2	3	4	5
			Flame	
Exp no	Sample volume µl	Number of impulses	K increment µM	K 10^{2} pmoles/cm² imp
43 a	202	5	100	4 5
41 a	165	10	48	1 0
44 a	261	10	100	3 3
44 b	277	10	122	4 3
42 a	252	10	163	5 3
42 c	308	10	117	4 7
45	298	10	160	6 2
43 b	421	20	200	5 3
				Mean 4 3

the C recording has sufficiently high sensitivity to show the increment in ionic concentrations associated with a single action potential (*cf* Methods) Such a recording from an alga *in lake water is illustrated in Fig 9 It appears that the course of the* conductivity change is similar to that obtained by I_g recording in distilled water The change is seen to start immediately after the peak of the action potential and the maximum is followed by the characteristic return to a final lower plateau representing the ionic equilibration of the solution in the middle compartment A small component of intracellular origin may be involved in the C recording (*cf* Methods) and no definite conclusion can be drawn about the quantitative relations until this possibility has been ruled out

Under the prevailing experimental conditions the stability of the C recording is *not comparable to that of the* I_g *recording by which as illustrated above it is possible* to follow the stepwise increase in conductivity during hour long experiments This is due to the increased effect of temperature variations on the C recording under these conditions (*cf* Methods) the slightest air currents over the test chamber being reflected as shifts in the baseline However with adequate shielding of the test chamber against various disturbances the stability of the C recording in lake water can be expected to match that of the I_g recording in distilled water at corresponding degrees of sensitivity

The quantitative chemical determinations of the ions involved had to be limited *to* K^+, *the method used for Cl determinations being too inexact at the high initial* Cl concentration of lake water It appears from Table V that the average value of the K^+ efflux per cm² impulse is of the same order of magnitude being about twice as large as that for algae in distilled water (*cf* Tables III and IV) The values for individual algae in lake water were found to vary to a certain extent like those for algae in distilled water

Discussion

Measuring changes in ionic concentration by means of high frequency reflectometry has proved to be a method of sufficiently high sensitivity to record the ionic efflux from an alga during a single activation. In the main series of experiments advantage was taken of the capacity of the algae to maintain their excitability even when immersed in distilled water. Starting from distilled water as test solution made it possible to use the I_g recording as index of the extracellular concentration changes, a procedure offering certain advantages, as described above, and the low initial concentration of the test solution also facilitated the quantitative chemical determination of the ions involved. However, the reflectometric method could also be used to record the ionic efflux from algae in their natural lake water milieu, as shown in a series of comparative experiments. In this conductivity range the C recording is a very sensitive index of ionic changes. The shielding against intracellular effects was however less effective than in the case of I_g recording. The good stability of the I_g recording allowed of continuous monitoring of the ionic concentration during hour long experiments. With the present experimental set up a corresponding stability could not be attained by the C recording mainly because of the increased influence of temperature variations.

Judging from the recordings of the conductivity changes in distilled water and in lake water respectively, the time course of the ionic outflow from the alga seemed to be relatively independent of the outer chemical milieu. Quantitative variations might be expected in view of the difference in ionic concentration gradients across the membrane in the two kinds of external solutions. However, with respect to the gradients of the ions involved in excitation the change from lake water to distilled water is not as extreme as would appear from the high conductivity value of lake water. This value is mainly due to high concentrations of Ca^{2+} and HCO_3^- ions (*cf* Table I), whereas the concentrations of the ions involved in the activation process are relatively low. The K^+ value is only 50 μM and this value is in fact attained in the test compartment initially filled with distilled water after an average of five impulses (*cf* Table III). Also the normal value of Cl^- in lake water is comparatively small, 200 μM. The experimental results showed in fact that the K^+ efflux per cm^2 impulse was of a similar order of magnitude in the two types of external media, the average value for the algae in lake water although twice as high as for the algae in distilled water being still within the range of variations of the latter. This shows that the ionic processes were not seriously disturbed by keeping the algae in distilled water at the beginning of the experiments. Of interest in this connection is a suggestion by Umrath (1954) based on studies of resting and action potentials of algae in various media to the effect that the algae possess an active adaptation mechanism diminishing the ionic permeability of algae immersed in distilled water.

The following discussion will be concerned mainly with the results from experiments on algae in distilled water using the I_g recording.

The validity of the I_g value as an index on conductivity has been established in investigations on solutions of different compositions (unpublished). Apart from

the effects of the rise in temperature mentioned in Methods, an additional error might be introduced due to residual coupling between the probe and the cell. However, it could be shown that the shielding of the probe against effects due to potential fields extending into the algae was efficient. That the algae or the compartment as such did not exert any loading effect interfering with the measurements was also evidenced by the unchanged I_g value measured in the test solution after its transfer to another vessel.

The high sensitivity of the I_g recording in the low conductivity range in combination with good baseline stability made it possible to follow quantitative variations in ionic efflux from one impulse to another in a series of consecutive stimulations. In earlier investigations with tracer technique only average efflux values were obtained (*cf.* below). A correlation of the effluxes per cm^2 impulse to the amplitude and duration of the individual action potentials seems pertinent in a subsequent analysis.

The chemical analyses served in the first place to identify the ions accounting for the conductance change recorded by the reflectometer. No attempts were made to attain a very high degree of accuracy or to collect a material large enough for statistical treatment of the data. The finding of an efflux of K^+ and Cl^- during excitation of *Nitellopsis* but of no other ions in significant quantities to account for the conductivity changes is in agreement with earlier results on *Chara globularis* by Gaffey and Mullins (1958).

An excitation mechanism like that suggested for *Chara* by Gaffey and Mullins (*cf.* also Kishimoto 1964), in which an exit of Cl^- is the primary process in excitation, would be an interesting variant of the commonly assumed mechanism of depolarization by inward movement of positively charged ions (*cf.* Hodgkin 1964). It would imply that the observed conductance change in the extracellular fluid would reflect the whole ionic flux occurring during the excitation. Whether there occurs an inward movement of Ca^{2+} during excitation of *Nitellopsis*, as has been claimed for *Chara australis* (Hope 1961, Findlay 1962) cannot be established by the present investigation. An essential role of Ca^{2+} in the excitation mechanism seems however less likely in view of the fact that the algae could produce action potentials in an external solution not containing any measurable amounts of Ca^{2+}. It cannot be excluded, however, that Ca^{2+} or some other cation in amounts adequate for the limited number of impulses elicited in the present experiments can be contained in the cell wall in spite of long lasting equilibration in distilled water before starting the experiment. The occurrence of a concomitant water transport during excitation, for which evidence has been presented by Teorell (1961) in studies on *Nitella* has also to be taken into account.

It seems of interest to compare the quantities of K^+ and Cl^- released per cm^2 impulse in our experiments with earlier findings based on tracer technique. In the absence of data from *Nitellopsis obtusa*, comparisons have to be made with other species. In tracer studies on *Nitella*, Mullins (1962) found an outflow of Cl^- varying from 0.8×10^3 to 3.0×10^3 picomole which is in good agreement with our findings

In studies on *Chara*, Gaffey and Mullins (1958) found values for the K^+ efflux of $3.1 \pm 1.1 \times 10^4$ picomole, thus about ten times higher than our values. The efflux of Cl^- was found to be somewhat smaller than the K^+ efflux, or $0.9 \pm 0.7 \times 10^4$ picomole, whereas judging from our preliminary series of experiments the Cl efflux seems to match the K^+ efflux in *Nitellopsis obtusa*. Further experiments on algae with normal resting outflow of Na^+ and Cl are however necessary before the relationship between the two fluxes can be determined more closely. As emphasized by Gaffey and Mullins (1958) and by Mullins (1962), their results imply an ionic outflow up to 5,000 times larger than that which according to the ionic theory is necessary to produce the observed potential change, and assuming similar electrical membrane constants in *Nitellopsis* the same discrepancy seems to apply also to this species.

In this connection the role of the cell wall as a diffusion barrier should be briefly discussed. It seems to be generally accepted that the algal cell wall possesses ion exchange properties (Gaffey and Mullins 1958, Dainty 1962), and it is possible that these may play a role under certain conditions. Thus it has been suggested that the cell wall might hold K^+ ions, forming an unstirred electrolyte layer only slowly equilibrating with the external fluid. However, the occurrence of a relatively fast outflow of ions without any subsequent slow leakage in the stimulus-free intervals, as indicated by the reflectometer recordings, seems to speak against the existence of a serious diffusion barrier in the cell wall.

The present investigation has proved the reflectometric method to be an adequate tool for quantitative measurements of minute ionic changes, but its most characteristic feature, the possibility of dynamic recording, has not been taken advantage of in this study. The examples given of such recordings suggest, however, that the reflectometric method of studying the time course of ionic changes during excitation may be a useful complement to the more elaborate method of fractionating tracer effluxes (Spyropoulos, Tasaki and Hayward 1961). Explorative experiments have shown that a comparison of the dynamic recordings of ionic effluxes with the changes in membrane conductance (*cf.* Cole and Curtis 1938) will also be informative and that a simultaneous application of I_g and C recordings will be of value in the further analysis of the temporal relationship between ionic effluxes and electric phenomena.

This work has been supported by grants from The Swedish Medical Research Council, the foundation Therese och Johan Anderssons minne and the foundation Gustaf och Tyra Svenssons minne.

We are indebted to Professor W. Rodhe, Limnologiska institutionen, Uppsala, Sweden, for his courtesy to put the resources of the limnological laboratory at the lake of Erken at our disposal for the collecting of the algal material, and to Fil. kand. L. Kronborg and Mr. B. Hjälm for their valuable assistance in this collecting.

References

BLINKS, L R, Some electrical properties of large plant cells In *Electrochemistry in Biology and Medicine*, John Wiley and Sons Inc, New York, Chapman and Hall Ltd, London 1955 p 187—212

COLE, K and H J CURTIS, Electric impedance of *Nitella* during activity *J gen Physiol* 1938 *22* 37—64

DAINTY, J, Ion transport and electrical potentials in plant cells *Ann Rev Plant Physiol* 1962 *13* 379—402

FINDLAY, G P, Calcium ions and the action potential in *Nitella* *Aust J Biol Sci* 1962 *15* 69—83

FORMAN, J and D J CRISP, The radio-frequency absorption spectra of solutions of electrolytes *Trans Faraday Soc* 1946 *42 A* 186—194

FURMAN, N H and G W LOW, JR, The use of the concentration cell in quantitative analysis I The estimation of small amounts of chloride in salts *J Amer chem Soc* 1935 *57* 1585—1591

GAFFEY, C T, and L J MULLINS, Ion fluxes during the action potential in *Chara* *J Physiol* (Lond) 1958 *144* 505—524

HAAPANEN, L, A high sensitivity capacitance-meter *Electronic Engng* 1962 *34* 183—185

HAAPANEN, L, Application of high frequency reflectometry for measurement of minute changes in electrolytic conductance *Acta physiol scand* 1967 *69* 37—50

HAAPANEN, L and C R SKOGLUND, Recording of net ionic flux during a single action potential in *Nitellopsis obtusa* *Acta physiol scand* 1963 *59* 297—298

HAAPANEN, L and C. R SKOGLUND, Application of high frequency reflectometry for dynamic

[illegible]

an electrical conductivity probe *A I Ch E Journal* 1960 *6* 682—685

MACROBBIE, E A C and J DAINTY, Ion transport in *Nitellopsis obtusa* *J gen Physiol* 1958 *42* 335—353

MALMSTADT, H V and J D WINEFORDNER Precision null point potentiometry A simple, rapid and accurate method for low concentration chloride determinations *Analyt chim acta* 1959 *20* 283—291

MULLINS L J, Efflux of chloride ions during the action potential of *Nitella* *Nature* (Lond) 1962 *196* 986—987

SJÖSTRAND U Analysis of ionic tracer movements during single heart cycles *Acta physiol scand* 1964 *61* Suppl 227

SPYROPOULOS C S I TASAKI and G HAYWARD, Fractionation of tracer effluxes during the action potential *Science* 1961 *133* 2064—2065

TEORELL T An analysis of the current voltage relationship in excitable *Nitella* cells *Acta physiol scand* 1961 *53* 1—6

UMRATH K, Über die elektrische Polarisierbarkeit von *Nitella mucronata* und *Nitella opaca* *Protoplasma* 1954 *43* 237—252

Acta physiol. scand. 1967. 69. 69—78

From The Department of Physiology, University of Goteborg Sweden

The Relation Between Blood Flow in an Isolated Muscle Measured with the Xe^{133} Clearance and a Direct Recording Technique

By

I Kjellmer, I Lindbjerg[1], I Přerovský[2], and H Tonnesen[3]

Received 7 July 1966

Abstract

Kjellmer, I , I Lindbjerg, I Přerovský and H Tonnesen *The relation between blood flow in an isolated muscle measured with the* Xe^{133} *clearance and the direct recording technique* Acta physiol scand 1967 *69* 69—78

To allow a critical evaluation of the Xe^{133} clearance technique this method was compared with directly metered blood flow in the isolated gastrocnemius muscle of cats at different flow levels between rest and maximum exercise dilatation Whether the Xe^{133} was applied by close intra arterial injection or intramuscularly the clearance curve had a multiexponential course This was ascribed partly to local variations of the affinity of the tissues to the gas but mainly to an uneven perf[illegible]on of the m[illegible]cle When [illegible]

is ascribed to the different microcirculatory conditions created by the two modes of injections The relative volume of the well perfused part of the tissue was estimated by noting at which level of activity the curve deviated from the first phase of rapid clearance The well perfused fraction increased at higher blood flows It is concluded that the Xe^{133} clearance technique may give valuable information about the local circulation provided the limitations of the method are considered

Among the techniques for recording local blood flow the method based on measurements of the clearance of locally deposited indicators has attracted rising attention in recent years The theoretical basis of the method was given by Kety (1949) and the advantage of using Xe^{133} as the tracer has been discussed by Lassen, Lindbjerg and Munck (1964 The technique has proven valuable in clinical routine work for the investigation of patients with complaints of intermittent claudication (see *e g* Lassen *et al* 1964 Tonnesen 1965) A basic assumption for calculating the capillary blood flow from the observed wash out curve of the Xe^{133} is that there is

Present address
[1] Glostrup Hospital Copenhagen Denmark
[2] Institute for Cardiovascular Research Prague Czeckoslovakia
[3] Institute of Medical Physiology A University of Copenhagen Denmark

complete diffusion equilibrium between tissue and blood at all levels of blood flow The rate of removal of Xe^{133} from the tissue can then be expected to follow a monoexponential course in a homogeneously perfused tissue

The present study was prompted by some unexpected findings of disproportion between the blood flow calculated from Xe^{133} clearance and the directly recorded blood flow in the skinned hindlegs of dogs (Prerovsky, Hlavova and Vavrejn 1965) It was therefore considered indicated to compare the Xe clearance method with the direct recording technique in a homogeneous tissue where the blood flow could be varied within a wide range and could be kept constant during a long period at each flow level The isolated gastrocnemius preparation of cats where steady state vasodilatation was induced by electrically provoked contractions was considered suitable for this purpose

Methods

Experiments were performed on 12 cats weighing 2 2—4 7 kg After ether induction chloralose 70 mg/kg was given i v and a tracheal cannula was inserted The body temperature was kept

and the lower leg was completely removed at the knee joint Thus the gastrocnemius was left with intact connections to the femur and with the normal blood supply Great care was taken to ligate all small vessels so that only the main popliteal vessels were left The muscle was suspended between the fixed femur and a stand to which the Achilles tendon was fastened so that the muscle

operating an ordinate writer The flow meter was calibrated at each new flow level by means of a graduated cylinder stopwatch The blood pressure was measured from the contralateral femoral artery Intra arterial injections were given through a thin polyethylene catheter inserted through a side branch of the ipsilateral femoral artery

The peripheral end of the severed sciatic nerve was placed on bipolar silver electrodes and stimulated with supramaximal square wave pulses of 0 1 msec duration and 3—5 volts from a Grass stimulator model S4 The frequency of stimulation was varied between 0 5 and 6 imp sec

The isotope used was Xe^{133} dissolved in 0 9 per cent saline (The Radiochemical Centre Amersham Engl) to an activity of 1—2 mC/ml During i a injections 0 1—0 3 ml of this solution was rapidly (1—2 sec)
injected through
muscle bellies of t
part of the muscle
mize back diffusion of the tracer into the needle track

The activity was recorded with a 1 1/2 × 2 thallium activated NaI crystal collimated to see the whole muscle The output was fed into a scaler unit (Packard Tricarb) and from there split into two recording systems one logarithmic ratemeter (Packard model 310A) and one linear inte

rates The calculations are described by *e g* Lassen *et al* (1964 A partition coefficient of 0 for Xe^{133} between muscle tissue and blood was used (cf Conn 1961)

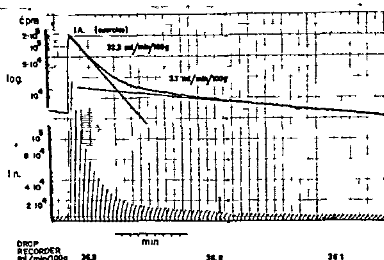


Fig 1 Externally recorded activity after intra arterial injection of Xe^{133} during muscular exercise Upper tracing shows the signal from the log ratemeter lower tracing signal from linear integrator Numerical values from simultaneously measured drop counter flows are given in the bottom line

Results

Intra arterial injections After i a injection of the isotope a clearance curve was obtained that was invariably multiexponential The initial slope could, however, be approximated to a straight line down to a level of radioactivity that corresponded to between 10 and 75 per cent of the initial activity Fig 1 gives a representative example of the shape of the clearance curve The figure shows the logarithmic and the linear records of the wash out curve while blood flow was stable during a period of muscular exercise When the blood flow in this experiment is calculated from the tangent of the clearance curve the initial part of the curve gives a flow rate close to the one recorded directly, while the latter part of the curve corresponds to a value ten times lower

In general the calculated blood flow based on the initial phase corresponded closely to the total blood flow measured with the drop counter technique Both blood flows varied with the level of muscle activity with a correlation coefficient of 0 95 ($P < 0\ 001$) The slope of the regression line was 0 93 *i e* the blood flow measured with the Xe^{133} method averaged 93 per cent of the total blood flow Fig 2 shows the comparison between the two methods

Blood flow was also calculated according to Zierler (1965) This method gives blood flow per unit volume from the ratio between peak activity value and total area under the curve Calculated in this way the blood flow was in all cases somewhat lower and averaged 80 per cent of the total blood flow

When the level of muscular activity was increased some time after the injection was given and after some of the Xe^{133} had been cleared the Xe^{133} clearance increased much less than the total blood flow Therefore the comparison in Fig 2 is

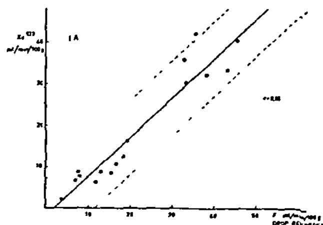


Fig 2 Relation between Xe^{133} clearance calculated from the initial portion of the washout curve after intra-arterial injections of the tracer, plotted against metered blood flow Regression line ± 2 SD is given and was calculated with the method of least squares Equation: y = 0 93 x —1 3

solely on experiments where the injection was given after the blood flow had reached a steady state and where the first few minutes of the clearance curve could be used for calculations

Intramuscular injections The Xe^{133} clearance curves obtained after local intramuscular injections of the tracer were usually multiexponential In 5 of 24 expts the curve was monoexponential almost down to background level, but in the others the semilogarithmic plot deviated from a straight line at a level of between 5 and 60 per cent of the initial activity

When the initial straight line was used for estimating the blood flow a correlation coefficient of 0 82 (P < 0 001) was found between the results of both methods The

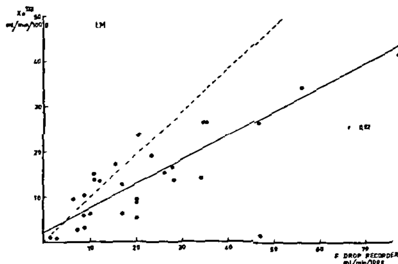


Fig 3 Relation between Xe^{133} clearance calculated from the initial portion of the washout curve after intramuscular injection of the tracer, plotted against metered blood flow Regression line calculated with the method of least squares is given according to equation y 0 62 x — 1 52 Dashed line = line of identity

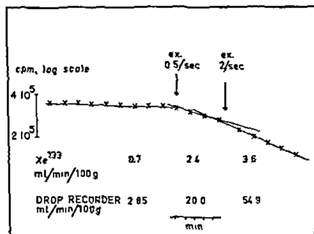


Fig 4 Xe[133] clearance after intramuscular injection of the tracer is shown during rest and during moderate and heavy exercise (0 5 and 2 contractions/sec, respectively) The bottom line gives the values for simultaneously obtained drop counter flow

slope of the regression line is 0 62, which implies that on the average the Xe[133] clearance amounted to only 62 per cent of the total blood flow Fig 3 shows the relation between the two methods Also in this case comparison is based only on clearance values during the first few minutes after injection Especially when recordings were performed late during the clearance period changes of muscular activity brought about much greater changes of total blood flow than of the rate of Xe[133] clearance, as demonstrated by Fig 4 Here the blood flow was increased to 55 ml/min $\times$ 100 g late during the clearance period by means of intense muscular exercise At the same time, however, the blood flow measured from Xe[133] clearance only increased to 3 6 ml/min $\times$ 100 g

The shape of the clearance curve varied systematically with the flow rate — the curve resembling more closely a monoexponential clearance at high blood flows This is demonstrated in Fig 5 where Xe[133] clearance curves are given from one muscle both during rest and exercise In both cases the initial slope of the semilogarithmic plot is linear However, at rest and at low blood flow the plot deviates from the straight line when about 50 per cent of the isotope is left in the tissue, while at higher blood flow during exercise the linearity is extended down to some 20 per cent remaining activity Fig 6 gives the results from all experiments The clearance rate calculated from the linear phase is plotted against the remaining activity at the level where the clearance curve leaves the straight line At resting blood flows the deviation from linearity in the semilogarithmic plot occurred while activity was still at 30—70 per cent of the initial level, while at maximal dilatation the clearance curve did not deviate from linearity until values well below 30 per cent of the initial activity were attained

RIS A injection In 5 expts RISA was injected i a and the rate of disappearance was followed by external counting Essentially the same type of wash-out curve was obtained as with Xe[133], *i.e* a multiexponential downslope of the clearance curve

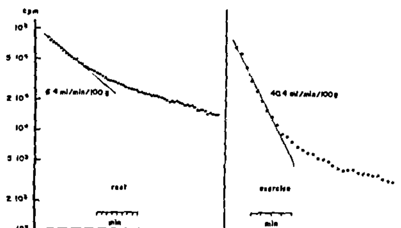


Fig 5 Clearance curves for intra arterially injected Xe^{133} plotted semilogarithmically Note that the time scales are different during rest and exercise The flow values given are calculated from the lines shown The corresponding metered flows were 12 0 ml/min/100 g during rest and 45 0 during exercise

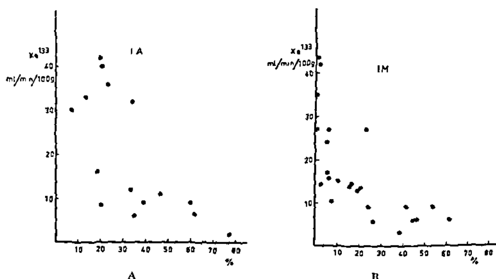


Fig 6 Xe^{133} clearance plotted against the fraction of the initial activity remaining in the tissue *when the semilog plot of the clearance curve deviated markedly from the monoexponential course* A) intra arterial and B) intramuscular injection of the tracer

Fat content Double determinations of fat content in three cat muscles gave values of 1 1—1 2, 1 3—1 4, and 1 0—1 0 per cent of the wet weight of the muscle tissue proper.

Discussion

The treatment of the clearance curves, as performed by *e g* Lassen, Lindbjerg and Munck (1964), is based on the assumption that the curve is a monoexponential function or can be divided into two or more monoexponential phases Our results indicate that in the present experimental situation the clearance curve is multi-

exponential but that the initial slope of the curve can be approximated to a straight line when plotted in a semilogarithmic system When a varying proportion of the indicator has been cleared (between 25 and 95 per cent of the initial concentration) the relative wash-out rate of the isotope becomes lower despite a stable total blood flow

Two different theoretical possibilities appear at hand to explain the deviation from the monoexponential curve Either it is due to an uneven binding of the isotope to various parts of the tissue or it depends on an uneven perfusion of the tissue The first possibility is in line with a preferential binding to fat tissue that has about ten times higher affinity for Xe^{133} than does muscle tissue (Conn 1961) But the fat content of the cat muscle is so low (about 1 per cent) that it can bind only a minor fraction of the injected isotope despite its high affinity for Xe

Despite the fact that a muscle appears to be a homogenous tissue, several observations point to the existence of an uneven perfusion of the muscle Renkin (1959) interpreted the results obtained on the exchange of K^{42} between blood and tissue in isolated gracilis muscle of dogs as evidence for an inhomogeneous microcirculation

Aukland, Bower and Berliner (1964) measured the clearance of inhaled hydrogen from the tissues by means of the hydrogen electrode and presented evidence that the myocardium of dogs is homogeneously perfused while the gracilis muscle is not

Freis *et al* (1957) injected T-1824 or Cr^{51}-tagged erythrocytes i a in the human forearm and determined the shape of the wash out curve obtained by venous sampling In this situation recirculation of indicator is minimal They obtained downslopes composed of more than one exponential phase and could also make likely that the results were not due to a mixture of blood from different tissues, suggesting that skeletal muscle was unevenly perfused

The present study included some experiments where the disappearance of i a administered RISA was followed Recirculation could be excluded and our results appear to corroborate the conclusion of Freis *et al*

It is of considerable interest that uneven perfusion of skeletal muscle occurs not only in isolated muscles of animals after extensive operations, but also is found in intact human muscles (Linde and Wahren 1965)

An uneven perfusion of the muscle may be visualized in different ways Barlow, Haigh and Walder (1961) discussed the possibility of a parallel circulation through the muscle tissue proper and the connective tissue of the muscle, *i e* a dual circulation There is also the possibility of uneven distribution of flow between several parts of the true muscle The present results which indicate that the best perfused muscle fractions increase in size at higher blood flows (Fig 6A and B) are in line with the latter view, namely that the increased muscular activity leads to an increased flow in certain, previously less well perfused parts

The fact that the muscle appears more homogeneously perfused at the highest flow rates Fig 6) may indicate that the clearance method, when applied to skeletal

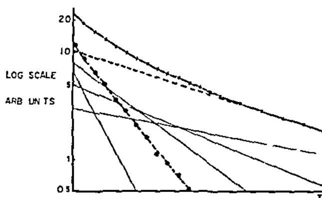


Fig 7 Constructed example of the graphical analysis of a clearance curve The imag ned tissue consists of four compartments perfused at different rates (thin lines) which will be recorded as the composite curve given by the crosses When th s curve is analyzed in the conventional way by subtracting the slowest component (dashed line) only one further monoexponential function will result (the filled circles)

muscle has its greatest merits during conditions of maximum dilatation This is also the clinical situation where the method has been tested in skeletal muscle and has proven valuable (Lassen Lindbjerg and Dahn 1965, Tonnesen 1965)

An uneven perfusion of the muscle would result in a wash out curve where the first part of the curve is representative of the whole tissue provided all compartments are in diffusion equilibrium with the blood, while the latter part of the clearance curve is dominated by the slower phases Thus the relation between total blood flow and clearance can be expected to be better when the first phase of the clearance curve is used than when the latter phases are taken into account This is clearly demonstrated by Fig 1 and Fig 4 The type of analysis used here is thus similar to the initial slope analysis of Ingvar and Lassen (1962) They provided evidence that the initial slope of the curve represents a weighted mean of the flows through all compartments provided their initial concentrations of tracer are the same

The present clearance curves have not been analyzed according to the method which subdivides the curve into two or more monoexponential components This is an approach which may appear meaningful in organs composed of different types of tissues or under other circumstances where a particular meaning can be ascribed to each subdivision of the curve In the skeletal muscle however it appears more reasonable to assume that the clearance curve is composed of a distribution of a multitude of components with regard to the architecture of the vascular bed

Fig 7 shows a constructed example that demonstrates that a clearance curve (the crosses) composed of four different components (the thin lines) may well be graphically resolved into only two compartments (the dashed lines) The figure stresses that the fact that a clearance curve may be analyzed into two compartments does not prove that these compartments exist It should also be noted that these two

compartments neither represent the slowest nor the fastest of the real compartments This potential fallacy of the graphical analysis was clearly demonstrated by Briscoe and Cournand (1959) in their examination of the helium wash-out curves from the lungs

Zierler (1965) has presented an alternate mode of analyzing the clearance curve His method requires knowledge of the peak activity and the area under the clearance curve and is free of assumptions regarding the shape of the washout curve When our curves were analyzed according to this method the calculated flows were about 15 per cent lower than with the alternate mode of calculation The results from the i a and i m injections were affected equally The percentage difference between the results from the two modes of calculation did not vary with the flow rate in our material

The blood flow values calculated from clearance curves after i a injections were better correlated with the total blood flows than the clearance values after i m injections In the first case the calculated blood flows averaged 93 per cent of the drop counter flows while they averaged only 62 per cent after intramuscular injections

It should be emphasized that the sources of error of the tissue clearance technique anatomical shunting, uneven distribution of blood flow incomplete diffusion equilibrium between tissue and blood, preferential tagging of areas with high partition coefficients, all tend to lower the blood flow values calculated from the clearance curves

The mode of tagging the tissues with isotopes apparently affects the results With i m injection there is the risk of depositing the whole amount of isotope in a place where the partition coefficient is different from that of muscle tissue or where the local circulation is slower, *e g* close to fascia or tendon However, in the isolated muscle used in the present experiments the exact position of the needle tip was readily determined and care was taken to inject directly into the fleshiest part of the muscle

The systematically low calculated blood flows after i m injections — even during intense hyperemia when virtually all capillaries should be perfused — demand, however some other explanation It seems reasonable to assume that during i a injection the tissues tagged with isotope are those parts immediately surrounding functioning capillaries The pathway for back diffusion would then be very short After i m tagging on the other hand, areas both close to and further away from open capillaries would be tagged and the mean diffusion distance must be longer in this situation The i m mode of injection also involves the possibility of local tissue damage and derangement of the microcirculation Furthermore, during i m depositions poorly perfused areas receive a proportionately larger part of the tracer than during i a injections Thus several factors might cooperate to create the difference in results between the two modes of injection

The implications of this study appear to be

that there is a statistically significant correlation between total blood flow and

blood flow calculated with this clearance technique, provided the initial portion of the clearance curve is used,

that a slowing of the washout of Xe^{133} from a single muscle occurs after some time — despite a constant blood flow, implying an uneven distribution of blood flow within the muscle,

that the muscle becomes more homogeneously perfused at maximum dilatation so that the washout curve then approximates a monoexponential function,

that intra arterial tagging gives blood flow values that approximate the total blood flow, and

that intramuscular tagging results in considerably lower blood flows but that changes of these are still well correlated with changes of total blood flow

References

AUKLAND K, B F BOWER and R W BERLINER Measurement of local blood flow with hydrogen gas. *Circulat Res* 1964 *14* 164—187

BARLOW T E, A L HAIGH and D N WALDER Evidence for two vascular pathways in skeletal muscle *Clin Sci* 1961 *20* 367—385

BRISCOE W A and A COURNAND Uneven ventilation of normal and diseased lungs *J appl Physiol* 1959 *14* 284—290

CONN H L. Equilibrium distribution of radioxenon in tissue xenon hemoglobin association curve *J appl Physiol* 1963 *16* 1065—1070

FREIS E D, H W SCHNAPER and L S LILIENFELD Rapid and slow components of the circulation of the human forearm *J clin Invest* 1957 *36* 245—253

INGVAR D H and N A LASSEN Regional blood flow of the cerebral cortex determined by krypton^{85} *Acta physiol scand* 1962 *54* 325—338

KETY S S Measurement of regional circulation by the local clearance of radioactive sodium *Amer Heart J* 1949 *38* 321—328

LASSEN N A, I F LINDBJERG and O MUNCK Measurement of blood flow through skeletal muscle by intramuscular injection of Xe^{133} *Lancet* 1964 *1* 686—689

LASSEN N A, I F LINDBJERG and I DAHN Validity of the Xenon^{133} method for measurement of muscle blood flow evaluated by simultaneous venous occlusion plethysmography *Circulat Res* 1965 *16* 287—293

LINDBJERG I F, A M ANDERSEN, O MUNCK and M JORGENSEN The fat content of leg muscle and its influence on the Xenon^{133} clearance method of blood flow measurement *Scand J clin Lab Invest* 1966 *18* In press.

LINDE B and J WAHREN Evidence of dual circulation in human forearm muscle during exercise *Scand J clin Lab Invest* 1965 *17* Suppl 86 164—165

PŘEROVSKÝ I, A HLAVOVA and B VAVREJN In *Radioisotopes in animal nutrition and physiology* Vienna 1965 631—641

RENKIN E M Transport of potassium 42 from blood to tissue in isolated mammalian skeletal muscle *Amer J Physiol* 1959 *197* 1205—1210

TONNESEN K H The blood flow through the calf muscle during rythmic contraction and in rest in patients with occlusive arterial disease measured by Xenon^{133} *Scand J clin Lab Invest* 1965 *17* 433—446

ZIERLER K Equations for measuring blood flow by external monitoring of radioisotopes *Circulat Res* 1965 *16* 309—321

Acta physiol. scand. 1967. 69. 79—86

From the Wihuri Research Institute, Helsinki, Finland

Incorporation in Vivo of Acetate-1-^{14}C into Liver and Serum Lipids in Hemorrhagic Lipemia in the Rat

By

E.-L. Hirvisalo and Antti Louhija

Received 22 July 1966

Abstract

Hirvisalo, E.-L. and A. Louhija. *Incorporation in vivo of acetate-1-^{14}C into liver and serum lipids in hemorrhagic lipemia in the rat.* Acta physiol. scand. 1967. *69*. 79—86.

[illegible]

Hemorrhagic lipemia is a distinct form of hyperlipidemia, which was described for the first time in 1909 by Boggs and Morris (for review of literature see Louhija 1965). Hyperlipidemia occurs after severe bleedings in many laboratory animals and in man. The serum becomes turbid, "lipemic", serum triglycerides increase markedly and serum cholesterol and phospholipids to a lesser degree. When the hemorrhagic anemia is corrected, the hyperlipidemia also subsides. Hemorrhagic lipemia is caused by the loss of red cells while the loss of blood plasma is of little, if any, significance. The most probable etiological factor is the anemic hypoxia (Starup 1934, 1937, Louhija 1965).

The exact developmental mechanism of hemorrhagic lipemia is not known. In a recent study some pertinent aspects were investigated (Louhija 1965). It was observed that in hemorrhagic lipemia there did not exist any signs of increased peripheral lipid mobilization or accelerated turnover of plasma free fatty acids in the liver. Thus, e.g., serum free fatty acid and glycerol concentrations fell during the development of hemorrhagic lipemia, and no increase in the production of free fatty acids was observed when adipose tissue of bled rats was incubated *in vitro*,

blood flow calculated with this clearance technique, provided the initial portion of the clearance curve is used,

that a slowing of the washout of Xe^{133} from a single muscle occurs after some time — despite a constant blood flow, implying an uneven distribution of blood flow within the muscle,

that the muscle becomes more homogeneously perfused at maximum dilatation so that the washout curve then approximates a monoexponential function,

that intra arterial tagging gives blood flow values that approximate the total blood flow, and

that intramuscular tagging results in considerably lower blood flows but that changes of these are still well correlated with changes of total blood flow

References

AUKLAND, K, B F BOWER and R W BERLINER, Measurement of local blood flow with hydrogen gas *Circulat Res* 1964 *14* 164—187

BARLOW, T E A L HAIGH and D N WALDER Evidence for two vascular pathways in skeletal muscle *Clin Sci* 1961 *20* 367—385

BRISCOE W A and A COURNAND Uneven ventilation of normal and diseased lungs *J appl Physiol* 1959 *14* 284—290

CONN, H L Equilibrium distribution of radioxenon in tissue xenon hemoglobin association curve *J appl Physiol* 1963 *16* 1065—1070

FREIS, E D H W SCHNAPER and L S LILIENFELD Rapid and slow components of the circulation of the human forearm *J clin Invest* 1957 *36* 245—253

INGVAR D H and N A LASSEN Regional blood flow of the cerebral cortex determined by krypton[85] *Acta physiol scand* 1962 *54* 325—338

KETY S S Measurement of regional circulation by the local clearance of radioactive sodium *Amer Heart J* 1949 *38* 321—328

LASSEN N A I F LINDBJERG and O MUNCK Measurement of blood flow through skeletal muscle by intramuscular injection of Xe^{133} *Lancet* 1964 *I* 686—689

LASSEN N A I F LINDBJERG and I DAHN Validity of the $Xenon^{133}$ method for measurement of muscle blood flow evaluated by simultaneous venous occlusion plethysmography *Circulat Res* 1965 *16* 287—293

LINDBJERG I F A M ANDERSEN O MUNCK and M JORGENSEN The fat content of leg muscle and its influence on the $Xenon^{133}$ clearance method of blood flow measurement *Scand J clin Lab Invest* 1966 *18* In press

LINDE B and J WAHREN Evidence of dual circulation in human forearm muscle during exercise *Scand J clin Lab Invest* 1965 *17* Suppl 86 164—165

PŘEROVSKY I A HLAVOVA and B VAVREJN In *Radioisotopes in animal nutrition and physiology* Vienna 1965 631—641

RENKIN E M Transport of potassium 42 from blood to tissue in isolated mammalian skeletal muscle *Amer J Physiol* 1959 *197* 1205—1210

TONNESEN K H The blood flow through the calf muscle during rythmic contraction and in rest in patients with occlusive arterial disease measured by $Xenon^{133}$ *Scand J clin Lab Invest* 1965 *17* 433—446

ZIERLER, K, Equations for measuring blood flow by external monitoring of radioisotopes *Circulat Res* 1965 *16* 309—321

Acta physiol. scand. 1967. 69. 79—86

From the Wihuri Research Institute, Helsinki, Finland

Incorporation in Vivo of Acetate-1-^{14}C into Liver and Serum Lipids in Hemorrhagic Lipemia in the Rat

By

E.-L. Hirvisalo and Antti Louhija

Received 22 July 1966

Abstract

Hirvisalo, E.-L. and A. Louhija. *Incorporation in vivo of acetate-1-^{14}C into liver and serum lipids in hemorrhagic lipemia in the rat.* Acta physiol. scand. 1967. 69. 79—86.

Hemorrhagic lipemia is a distinct form of hyperlipidemia, which was described for the first time in 1909 by Boggs and Morris (for review of literature see Louhija 1965). Hyperlipidemia occurs after severe bleedings in many laboratory animals and in man. The serum becomes turbid, 'lipemic', serum triglycerides increase markedly and serum cholesterol and phospholipids to a lesser degree. When the hemorrhagic anemia is corrected, the hyperlipidemia also subsides. Hemorrhagic lipemia is caused by the loss of red cells while the loss of blood plasma is of little, if any, significance. The most probable etiological factor is the anemic hypoxia (Starup 1934, 1937, Louhija 1965).

The exact developmental mechanism of hemorrhagic lipemia is not known. In a recent study some pertinent aspects were investigated (Louhija 1965). It was observed that in hemorrhagic lipemia there did not exist any signs of increased peripheral lipid mobilization or accelerated turnover of plasma free fatty acids in the liver. Thus, e.g., serum free fatty acid and glycerol concentrations fell during the development of hemorrhagic lipemia, and no increase in the production of free fatty acids was observed when adipose tissue of bled rats was incubated *in vitro*,

as compared with sham-bled control rats Nor did the carcass fat concentration of bled rats differ from the normal Further it was observed that serum ketone bodies decreased during the development of hemorrhagic lipemia, and that after administration of albumin bound 1-^{14}C-palmitic acid the recovered radioactivities of liver lipid fractions during 3 hrs were the same in bled and sham bled control rats On the other hand, the rate of elimination of the label, which had re entered in esterified form into the blood, was slower in the rats with hemorrhagic lipemia than in the controls From this it is difficult to draw any conclusions concerning the rate of lipid release from the liver or the peripheral uptake, as the increased pool of esterified fatty acids itself accounts for a retarded disappearance of the label in this kind of an experiment

If increased triglyceride and lipoprotein synthesis in the liver (with or without concomitant decrease of peripheral lipid elimination) is a significant factor in the genesis of hemorrhagic lipemia, then the fatty acids must necessarily be derived from synthesis *de novo*, since increased peripheral fatty acid mobilization and uptake in the liver has been ruled out In order to obtain an idea of the activity of the *de novo* synthesis of fatty acids in hemorrhagic lipemia a study was made in which the incorporation of i p administered sodium acetate -1-^{14}C into liver and serum lipids was investigated in rats rendered hyperlipidemic by repeated bleedings, and in sham bled control rats

Materials and methods

Experimental procedures 35 male rats of the Sprague Dawley strain were used They weighed 180—230 g at the beginning of the experiments They were fed on a commercial rat chow Food and water was given *ad libitum* and the actual consumption was measured daily

The hemorrhagic anemia was produced by daily bleedings under Nembutal[R] anesthesia The tip of the tail was cut and an amount of blood corresponding to 2 ml — 10 % per 100 g b w was

[illegible]

third day of bleeding onwards except on the final day when all rats were fed *ad libitum*

The details of the bleeding technique the food consumption the changes in the body weight of the animals and the degree and characteristics of the developing anemia and of the ensuing hyperlipidemia have been described previously (Louhija 1965)

On the 7th day from the beginning of the bleedings about 24 hrs after the last (6th) bleeding either 7.5 or 18 μc of sodium acetate 1 ^{14}C specific activity 29.0 mc/mmole The Radiochemical Centre Amersham dissolved in 0.4 ml of physiological saline was injected i p After either 45 min or 3 hrs the rats were killed under ether anesthesia by withdrawing as much blood as possible by cutting the abdominal aorta The liver was removed rinsed carefully in water blotted dry weighed and homogenized in chloroform methanol 2 1 The extraction of lipids was carried out according to Folch *et al* (1957 After coagulation the collected blood was centrifuged and the serum lipids were extracted by the same Folch procedure Samples from the lower (chloroform) phase of the Folch extract were taken to dryness under nitrogen atmosphere and dissolved in chloroform Further analyses were made from this solution.

[illegible] methyl esters

[illegible] is taken

TABLE I Blood hemoglobin and hematocrit values and serum triglyceride and liver total fatty acid concentrations in rats bled about 2 ml per 100 g of body weight daily for 6 days, and of sham bled control rats

		Sham bled control rats n = 17	Bled rats, n = 18 In the beginning of daily bleedings	Bled rats, n = 18 After 6 daily bleedings
Hemoglobin	Mean	13 5	13 6	5.2
g/100 ml	S D	1 6	1 0	0 30
Hematocrit	Mean	42 4	40 3	22 4
%	S D	4 1	3 5	1 9
Serum triglycerides	Mean		34	67
mg/100 ml	Range		*16—52*	*31—126*
(skew distribution)				(after 5 daily bleedings)
Liver total fatty acids	Mean	133		157
µeq/g (wet weight)	S D	10 9		16 9

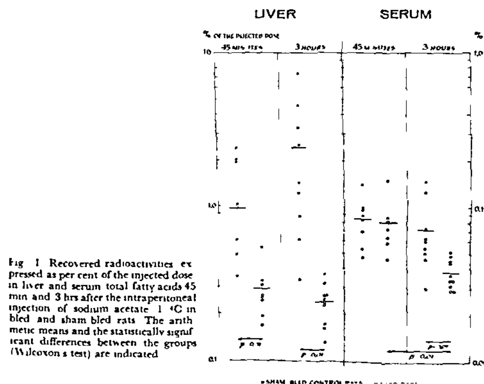


Fig 1 Recovered radioactivities expressed as per cent of the injected dose in liver and serum total fatty acids 45 min and 3 hrs after the intraperitoneal injection of sodium acetate 1 ¹⁴C in bled and sham bled rats The arithmetic means and the statistically significant differences between the groups (Wilcoxon s test) are indicated

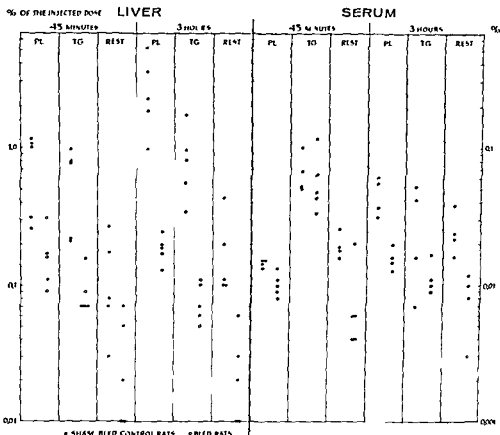


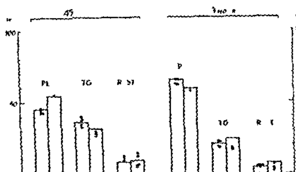
Fig 2 The distribution of radioactivity expressed as per cent of the injected dose in liver and serum lipid fractions (separated by thin layer chromatography) 45 min and 3 hrs after the i p injection of sodium acetate 1 ^{14}C in bled and sham bled rats PL = phospholipids TG = triglycerides Rest = all other lipids together

Determination of serum triglycerides The serum triglyceride concentration was determined according to Van Handel (1961)

Thin layer chromatography Liver and serum lipids were separated by TLC (Mangold 1962) Glass plates 20 × 20 cm with a 0 5 mm layer of silica gel G (Kieselgel G nach Stahl E Merck AG Darmstadt) were used and the sample was pipetted to a front of 10 cm The plates were developed 10 cm with petroleum ether ether acetic acid 90 10 1 (v/v/v) and standard reference spots were developed on the same plate The lipids were made visible with aqueous Rodamine 6 G solution (0 008 %) The silicic acid from the plates was then scraped off in three fractions containing phospholipids triglycerides and all the rest respectively The lipids were eluted with chloroform methanol water 64 32 4 (v/v/v) (phospholipids) or chloroform methanol 1 1 (v/v) (other fractions) and evaporated to dryness The recovery of the label was 92 per cent on an average (range 85—99 per cent) for liver lipids and 84 per cent (71—94 per cent) for serum lipids

Radioactivity measurements Radioactivity was measured from liver and serum fatty acid methyl esters after acid methanolysis and from liver and serum lipid fractions separated by thin layer chromatography as described above The samples were transferred into counting vials taken to dryness dissolved in toluene with 0 3 % PPO and 0 03 % POPOP and measured in a Tricarb

Blood hemoglobin was measured as oxyhemoglobin by
₀ ammonia water and reading the colour photometrically
ı derived from a standard curve prepared by using the commercial standard hemoglobin solution Hemotrol (Clinton Laboratories Los Angeles) Hematocrit values were determined by using the heparinized tubes and micro hematocrit centrifuge of Hawksley & Sons Ltd London



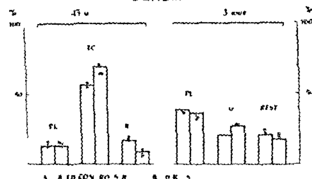


Fig 3

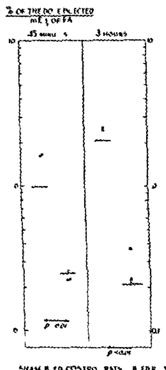


Fig 4

Fig 3 Relative distribution of radioactivity as per cent of the total recovery in liver and serum lipid fractions (separated by thin layer chromatography) 45 min and 3 hrs after the ip injection of sodium acetate 1-^{14}C in bled and sham bled rats PL = phospholipids TG = triglycerides Rest = all other lipids together

Fig 4 Specific activity of liver total fatty acids 45 min and 3 hrs after the ip injection of sodium acetate 1-^{14}C in bled and sham bled rats The arithmetic means and the statistically significant differences between the groups (Wilcoxon's test) are indicated

Results

The mean blood hemoglobin values and the mean serum triglyceride and liver total fatty acid concentrations in bled and sham bled rats appear from Table I The daily bleedings of about 2 per cent of body weight did not cause quite as severe anemia as in previous experiments (Louhija 1965) Consequently the ensuing hyperlipidemia was not severe the serum triglyceride values being on an average only twice their initial values ($p < 0.001$ Student's t test) after 5 days bleeding A slight tendency to fat accumulation in the liver was observed in the bled rats the mean liver total fatty acid concentration of these rats (157 ± 16 9 μeq/g) was somewhat higher than that of the sham bled controls (133 ± 10 9 μeq/g) the difference being statistically significant ($p < 0.001$ Student's t test)

The recovered radioactivities of liver and serum total fatty acids expressed as per cent of the injected dose are shown in Fig 1 The distribution of the label in liver

and serum phospholipids, triglycerides, and all other lipids together ("rest"), expressed as per cent of the injected dose, in 10 bled and 10 control rats, are presented in Fig 2 When extrapolating the total activities from the measured serum samples, the total serum volume was estimated at 4 4 ml/100 g of b w in the controls and at 5 5 ml/100 g of b w in the anemic rats (Louhija 1965)

It can be seen, that significantly less acetate was incorporated into liver total fatty acids in the bled rats than in the sham-bled controls both after 45 min and 3 hrs In serum total fatty acids there was no difference between the total activities after 45 min, but after 3 hrs the activity of serum total fatty acids was significantly lower in the bled rats than in the sham bled controls Concerning the recovery of the label from the different fractions it can be seen that except for serum triglycerides where about equal absolute recoveries were found, the absolute recovery of the label was lower in bled rats in all the other lipid fractions of both liver and serum There were, however, no remarkable differences in the relative distribution of the label in the lipid fractions when expressed as per cent of the total recovery, as appears from Fig 3

The specific activity of liver total fatty acids, calculated as per cent of the injected dose per meq of fatty acid, is shown in Fig 4 It can be seen that, consistently, the specific activity was significantly lower in the livers of the bled rats than in those of the sham bled controls The same result would naturally have been obtained for the serum specific activities, but serum total fatty acid determinations were not made

Discussion

Lipogenesis is very sensitive to the nutritional state, to the eating pattern, and to the dietary fat content (for comprehensive review see *e g* Masoro 1962) In the present experiment care was taken to ensure identical food consumption and eating patterns of the bled rats on the one hand, and the control rats on the other hand On the last day until the injection of radioacetate all the rats were allowed to eat *ad libitum*

The incorporation of i p injected radioacetate into lipids of various tissues is rapid Maximum recovery of the label from fatty acids and cholesterol fractions of total body lipids and lipids of liver and various other tissues is attained in less than 20 min in the rat, and significant changes from this maximum do not occur during the following 4 hrs (Van Bruggen *et al* 1953) In the present experiment two arbitrary points of this period of maximum incorporation were chosen (45 min and 3 hrs)

It was observed that the incorporation of radioacetate into liver and serum fatty acids was markedly reduced in hemorrhagic lipemia From this it cannot be concluded right off that there is a reduction of lipogenesis in hemorrhagic lipemia, because it is not known whether the acetate pool is the same in bled and control rats and, *further*, the acetate must be distributed to the tissues and activated to acetyl CoA before entering into fatty acid synthesis Lipid labelling from i p administered radioacetate has been widely used, however, as an indicator of the rate of lipid synthesis and has, in fact, given results compatible with those obtained by other methods This is true

concerning for instance the effects of fasting and dietary pattern and of diabetes on lipogenesis (reviewed *e g* by Langdon 1960 and Masoro 1962)

Further, the fact that the label tends to disappear faster from both liver and serum lipids in the bled rats than in the controls clearly corroborates the assumption that lipid synthesis from acetate cannot be increased in the bled rats On the contrary, a slower disappearance than in the sham bled controls would have been expected if the rate of acetate incorporation were increased or even unchanged in the bled rats, as it must be taken into account that the pool of esterified fatty acids, especially of triglycerides, in liver and serum is markedly increased in bled rats In fact, it has been shown previously, that the rate of disappearance from the plasma of re esterified radiopalmitate is slower in bled rats than in sham bled controls (Louhija 1965)

After injection of labelled palmitic acid the label appears sooner and attains a higher level in liver triglycerides than in the phospholipids, and is also more rapidly eliminated from the triglycerides The same relations hold for plasma lipids, so that initially the main portion of the recirculating label is recovered as triglycerides but gradually *the phospholipids come to contain the main part of the plasma label* (Olivecrona 1962) The course of lipid labelling from 1 ^{14}C-palmitic acid is similar in hemorrhagic lipemia and does not differ from sham bled control rats in this respect (Louhija 1965) The present results show that the course of events is, as could be anticipated, quite the same when the label in liver and serum lipids is derived from radioacetate In spite of the differences in the total activities of liver and serum fatty acids there are no noteworthy differences between the bled and sham bled groups in the relative distribution of the label into liver and serum triglycerides and phospholipids Evans and Norcia (1964) have found in control hyperthyroid and hypothyroid rats still greater a proportion of the label to be incorporated into the phospholipid fraction The total recovery of the label from liver lipids one hour after injection of the radioacetate was between 1 80 and 1/200 and this is comparable to the present results on sham bled control rats

It would seem on the basis of the present experiments that the lipogenesis from acetate is decreased rather than increased in hemorrhagic lipemia Increased *de novo* synthesis of fats can in all probability thus be ruled out as a cause of this form of hyperlipidemia Since an increased peripheral mobilization of lipids has been ruled out in previous studies, it would seem that the only remaining possibility is that hemorrhagic lipemia arises through retarded elimination of the lipids from the circulation

References

Boggs T R and R S Morris Experimental lipemia in rabbits *J exp Med* 1909 *11* 553—560

Evans J D and L N Norcia Incorporation of acetate C^{14} into individual fatty acids of rat liver triglycerides and phospholipids *J Lipid Res* 1964 *5* 395—401

Folch J M Lees and G H Sloane Stanley A simple method for the isolation and purification of total lipides from animal tissues *J biol Chem* 1957 226 497 509

Langdon R G Hormonal regulation of fatty acid metabolism In *Bloch K Lipide Metabolism John Wiley & Sons New York London* 1960 238 290

Louhija A Metabolic studies on haemorrhagic lipaemia in the rat *Ann Med exp Fenn* 1965 43 Suppl ? 73 pp

MANGOLD H K

M

Ol

RENKONEN O A note on spectrophotometric determination of acyl ester groups in lipids *Biochim biophys Acta* (Amst) 1961 *54* 361—362

STARUP U Einige Untersuchungen uber die hämorrhagische Lipämie *Biochem Z* 1934 *270* 74—92

STARUP U Undersogelser over experimentel hyperlipaemi Thesis *Levin & Munksgaard—Ejnar Munksgaard Copenhagen* 1937 166 pp

VAN

Acta physiol. scand. 1967. 69. 87—94

From the Department of Pharmacology, University of Goteborg, Sweden

An Improved Method for the Fluorimetric Determination of 5-Hydroxytryptamine in Tissues

By

NILS-ERIK ANDEN and TOR MAGNUSSON

Received 2 August 1966

Abstract

ANDEN, N.-E. and T. MAGNUSSON. *An improved method for the fluorimetric determination of 5-hydroxytryptamine in tissues.* Acta physiol. scand. 1967. *69*. 87—94.

In the method described where the fluorescence of 5-hydroxytryptamine (5-HT) is read in 3 N HCl, tissue blanks are produced by means of UV irradiation in the presence of potassium ferricyanide. This treatment destroys only the 5-HT but no other substance fluorescing at 295/545 mμ. With this new type of bl[illegible]

[illegible]

tetraacetate. The recovery and reproducibility are satisfactory.

Two different fluorimetric methods for the determination of 5-hydroxytryptamine (5-HT) in tissues have been described: one method is based on determining the native fluorescence of 5-HT in 3 N HCl (Udenfriend, Bogdanski and Weissbach 1955, Bogdanski *et al.* 1956) and the other method is based on the reaction between 5-HT and ninhydrin (Vanable 1963, Snyder, Axelrod and Zweig 1965). The amount of 5-HT should be calculated from the difference between the reading values of the sample and the tissue blank but the use of a tissue blank has not been described in either of the two methods mentioned above. The blank value may not be of great importance when the 5-HT content is high. However, when the amount of 5-HT is low, as is found in the brain after lesions or reserpine treatment, it is essential to have reliable tissue blank values. We have observed that certain batches of concentrated HCl which contain Fe^{+++}-ion as an impurity can cause a rapid destruction of 5-HT by the activation light in the spectrophotofluorimeter. This finding has allowed us to develop a method for the production of tissue blanks. Furthermore, the sensitivity of the method could be increased by reducing the volume of the reagents and the eluate. The stability of the 5-HT in tissue extracts has also been improved

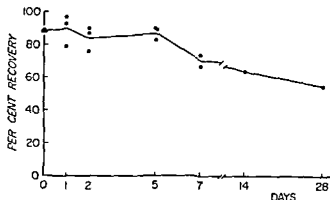


Fig. 1. Stability of 5-hydroxytryptamine in extracts from brains of reserpine treated rabbits. 5-Hydroxytryptamine, ascorbic acid and EDTA were added to 0.4 N perchloric acid extracts. The pH of the extracts was adjusted to 5—6 and stored at —20° C.

Preparation of extracts

Immediately after sacrificing the animals the tissue samples are placed in centrifuge tubes containing ice cold 0.4 N perchloric acid (4—5 ml per g tissue), ascorbic acid (2 %, 0.1 ml per 10 ml) and EDTA (disodium ethylenediamine tetraacetate, 10 %, 0.2 ml per 10 ml). The tissue is homogenized by means of an "Ultra Turrax" homogenizer and the homogenate is centrifuged at about 10,000 g for 10 min at 0° C. The supernatant is filtered and the residue is re-extracted with 0.4 N perchloric acid (3—4 ml per g tissue) containing ascorbic acid and EDTA. The pH of the combined filtrates is adjusted to 5—6 with 5 N potassium carbonate using brom phenol blue as an indicator. The extract may be stored at —20° C up to 5 days.

Comments. The preparation of the extracts is essentially that described by Bertler, Carlsson and Rosengren (1958) for catecholamines and adopted by Bertler (1961) for 5-HT. The stability of 5-HT in 0.4 N perchloric acid extracts is poor but, as reported by Gunne (1963), it can be markedly increased if the pH is adjusted almost to neutral. Since ascorbic acid can prevent the destruction of 5-HT in the presence of Fe^{+++} ions (see below), this substance and a metal chelating agent, EDTA, are added to the perchloric acid. The stability of 5-HT added to extracts from the brains of three rabbits pretreated with reserpine (2 + 1 mg/kg i.v. 24 and 12 hrs before sacrifice) is shown in Fig. 1. The supernatant from each brain was divided in four or more equal parts and 0.5 μg 5-HT was added to each fraction. One portion from each brain was analyzed immediately and an average recovery of 88 per cent was obtained. The other portions were stored at —20° C for different times before the determination. The recovery was unchanged during the first 5 days, the longest storage period used.

Experiments were performed to check if the simplified extraction procedure of Bertler, Carlsson and Rosengren (1958) can also be used for 5-HT (Table I). The 5-HT in the brains of 5 untreated rabbits was extracted 5 times with 30 ml 0.4 N perchloric acid containing 0.3 ml 2 per cent ascorbic acid and 0.6 ml 10 per cent EDTA. The content of 5-HT in the supernatant from each of the 5 extracts was determined. The values obtained were compared to those calculated on the assumptions that the water content of the brain is 75 per cent and that the 5-HT is present

TABLE I The content of 5-hydroxytryptamine in the supernatants by 5 successive extractions of a single rabbit brain The figures are given as per cent of total

		Extraction no				
		I	II	III	IV	V
Brain 1[1]	Found	72	18	6	3	1
	Calculated	76	18	4	2	0
Brain 2	Found	71	19	7	2	1
	Calculated	74	19	5	2	0
Brain 3	Found	76	18	5	1	0
	Calculated	77	17	4	2	0
Brain 4	Found	75	17	6	2	0
	Calculated	75	16	8	1	0
Brain 5	Found	77	16	5	2	0
	Calculated	74	18	6	2	0

[1] Example Brain 1 weighed 8 11 g and would, thus contain 6 1 ml water The total volume of water in the homogenate was 30 + 0 3 + 0 6 + 6 1 = 37 0 ml The volume of the supernatant obtained after extraction I was 28 ml or 76 per cent of the total volume of water in the homogenate The supernatant was found to contain 72 per cent of the total amount of 5 hydroxytryptamine recovered from all the extracts

only in the water phase and evenly distributed in this There was a satisfactory agreement between the found and calculated values However, re extraction is usually performed when the amount of 5-HT is small since this procedure considerably increases the quantity recovered for the final estimation

The column step

A weak cation exchanger, Amberlite CG 50, type I (100—200 mesh) is used for the purification and the concentration of the extracts The column procedure is similar to that described by Bertler (1961) The resin is washed several times with dilute HCl and those particles which do not settle within 10 min are decanted The resin is transferred to a 50 mm long glass tube (inner diameter 4 5 mm) the tip of which is filled with glass wool The column is pretreated in the following way

1) 20 ml 2 N HCl
2) Redistilled water to remove the acid
3) 20 ml 0 1 M phosphate buffer, pH 6 5
4) 3 ml redistilled water

After this treatment the resin has a length of 35 mm

The frozen extract is thawed, the precipitated potassium perchlorate is centrifuged at 0° C and the supernate is put on a column at a rate not exceeding 0 5 ml per min For the ion exchange procedure the apparatus described by Bertler *et al* (1958) is employed After the passage of 3 ml redistilled water the column is washed with 10 ml 0 02 M phosphate buffer, pH 6 5, containing 0 2 per cent EDTA The buffer is

TABLE II Example of analysis of 5-hydroxytryptamine (5-HT) in the rat brain The spectra are shown in Fig 2

Reagents or solutions in ml	Standard	Reagent blank	Sample	Internal standard	Tissue blank
HCl, N	0.6	0.6	—	—	—
Ascorbic acid, 1%	0.1	0.1	0.1	0.1	—
$K_4Fe(CN)_6$ 0.025%	0.1	0.1	0.1	0.1	0.1
5-HT, 10 µg/ml	0.010	—	—	0.010	—
Eluate	—	—	0.6	0.6	0.6
HCl conc	0.4	0.4	0.4	0.4	0.4
UV irradiation 10 min	+	+	+	+	+
Ascorbic acid 1%	—	—	—	—	0.1
Arbitrary fluorescence units at 295/545 mµ	50	2	33.5	81.5	7

removed with 5 ml redistilled water Thereafter N HCl is put on the column at a rate not higher than 0.5 ml per min The first 0.7 ml of the eluate are discarded and the following 2.3 ml containing the 5-HT are collected

Comments The resin Amberlite CG 50, type I, has a more uniform particle size than Amberlite XE 64, the previously most used cation exchanger for 5-HT Thus it is easier to obtain a uniform rate of flow through the column Columns may be used up to ten times

We routinely examine the last drop of the first 0.7 ml of the eluate with an indicator paper and have never observed that the HCl will appear in it This is important because a large part of the 5-HT is eluated with the first drop of HCl When the amount of 5-HT is high, however, we collect all the 3 ml since it has not been observed that the first 0.7 ml contain any material disturbing the final determination

The largest tissue sample, which can be put on a column without any detectable breaking through is 5 g

Fluorimetry of 5-hydroxytryptamine

Immediately after elution an aliquot of 0.6 ml is taken for the assay This is put in a silica test tube together with 0.1 ml 1 per cent ascorbic acid and 0.1 ml 0.025 per cent potassium ferricyanide Then 0.4 ml concentrated HCl is added and the sample is irradiated for 10 min by means of a mercury lamp (Original Hanau, UV-tube NN 30/89, 15 cm distance between the UV-tube and the sample) The fluorescence is read within 30 min after irradiation in an Aminco Bowman spectrophotofluorimeter at the activation and fluorescence peaks (295/545 mµ, uncorrected instrumental values) A UV-filter is placed in the filter holder in front of the photocell This filter eliminates the second order light scatter peak at 590 mµ

A standard and a reagent blank are run in parallel with the sample A blank ("tissue blank") is treated in the same way as the sample except that the ascorbic

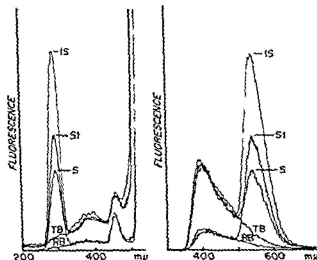


Fig 2 Activation (left) and fluorescence (right) spectra of rat brain samples The activation spectrum is recorded at a fluorescence wavelength of 545 mμ The fluorescence spectrum is recorded at an activating wavelength of 295 mμ St = 0 1 μg authentic 5 HT per 1 2 ml S = sample IS = internal standard (sample plus 0 1 μg 5-HT) TB = tissue blank RB = reagent blank The sample contains about 1/8 of the total amount of 5-HT in a single whole brain

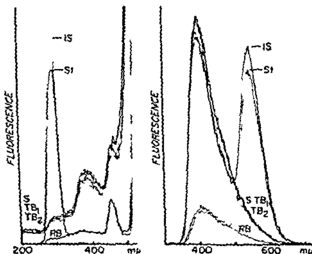


Fig 3 Comparison between tissue blanks and a sample from the brain of a reserpine treated rat St = 0 1 μg authentic 5-HT per 1 2 ml S = sample from the whole brain of a reserpine treated rat representing 1/5 of the tissue from a single whole brain IS = internal standard (sample plus 0 1 μg 5 HT) TB = tissue blank TB_1 = tissue blank to which 0 1 μg 5-HT was added before UV irradiation RB reagent blank

acid is added after the UV irradiation To control the possible interference by tissue *and resin material in the sample a known amount of 5 HT is added* to one portion of the eluate internal standard) and treated like the sample and the standard

The procedure is outlined in Table II The data obtained from a typical determination of 5 HT in a normal rat brain are also presented The activation and fluorescence spectra of this sample were recorded by means of a Moseley Autograf, model 135C and are shown in Fig 2

Comments The reliability of the tissue blanks produced was tested in the following way The brain of a rat injected 18 hours before with 10 mg/kg reserpine i p was treated as described above except that the entire 3 ml of eluate were collected Three

0 6 ml portions of eluate were taken for use as sample, internal standard and tissue blank, respectively A fourth portion was treated like the tissue blank except that 0 1 µg 5-HT was added before the UV irradiation The spectra are presented in Fig 3 and it is seen that the spectra of the two tissue blanks and the sample are nearly superimposed This shows that the treatment of the tissue blank removes all the 5 HT but no other material fluorescing at 545 mµ when activated at 295 mµ A reinjection of the same dose of reserpine to a rat did not produce any further reduction of the sample reading value On the other hand, in the rabbit brain even after a large dose of reserpine (2 mg/kg i v , 24 hrs) a small amount of 5 HT could still be detected An additional dose of 1 mg/kg 12 hrs later eliminated the small amount remaining

The fluorescence of 5 HT in 3 N HCl after the addition of ascorbic acid and potassium ferricyanide plus the UV irradiation was compared with that obtained without this treatment In these experiments it was necessary to use glass distilled 6 N HCl instead of concentrated HCl, otherwise the specific fluorescence would be reduced at a rapid rate probably due to the presence of Fe^{+++}-ions in the HCl The treatment reduced the fluorescence by about 20 per cent on the average and the ascorbic acid, the potassium ferricyanide and the UV irradiation seemed to contribute by approximately a third each

If the reading was performed a long time after the irradiation the values showed a tendency to be reduced, especially those of the standard Therefore the readings are performed within 30 min Under these conditions the agreement between the standard and the internal standard usually was good As a rule we accept only the determinations in which the difference in reading value between the internal standard and the sample is between 90 and 110 per cent of that between the standard and the reagent blank

Recovery and reproducibility

In 4 expts the brain homogenate of 2 untreated rats was divided into 4 equal parts A known amount of 5 HT (0 3 or 0 1 µg) was added to the homogenate of 2 of the 4 portions Analysis of 5 HT was thereafter carried out as described above The results are given in Table III The reading values of the duplicates agreed within a few per

TABLE III Recovery of 5 hydroxytryptamine (5 HT) from the rat brain

Sample no	No addition (duplicates) µg		Addition µg	Recovery of 5 HT added to homogenate 1 µg	per cent	homogenate 2 µg	per cent
1	0 29	0 29	0 30	0 24	80	0 26	87
2	0 30	0 36	0 30	0 25	83	0 28	93
3	0 30	0 27	0 10	0 08	80	0 07	70
4	0 29	0 32	0 10	0 08	80	0 07	70

cent The added 5 HT was recovered with about the same accuracy as when 5 HT was added to an extract (see below)

In 2 expts an extract was prepared from the brains of 2 rabbits pretreated with reserpine (2 + 1 mg/kg i v 24 and 12 hrs before sacrifice) as described above In both instances the extract was divided into 11 equal parts One portion served as a control of the reserpine effect on the 5 HT In the first experiment 0 5 μg was added to all 10 portions The mean recovery of 5 HT was 87 per cent with a standard deviation of 8 5 per cent In the second experiment the addition was 0 1 μg and the recovery was 86 ± 6 8 per cent (mean ± S D)

Sensitivity

With the present method typical activation and fluorescence spectra for 5 HT may be obtained with as little as 2 ng (1 ng = 10^{-9} g) 5 HT in a pure solution When tissue is included a somewhat larger amount is needed but 5 ng in the sample may be demonstrated, *i e* the extract must contain 25 ng The peak specific for 5 HT is easier to detect in the activation than in the fluorescence spectrum For a quantitative determination it is desirable to have at least 50 ng 5 HT in the extract

Specificity

All available 5 hydroxy- and 5 methoxy indole compounds have activation/fluorescence maxima at *295/545 mμ (Udenfriend et al 1955, Quay 1963)* In Table

TABLE IV The fluorescence the blank and the behaviour on a weak cation exchange column of some 5 OH and some 5 OCH_3 indole compounds

	[1]Relative fluorescence intensity of the		Peaks of act /fluor in mμ	After putting on a column (Amberlite CG 50 type I Na form) found in the
	[2]sample	[2] [3]blank		
5 HT	[4]50	5	295/545	eluate (3 ml N HCl)
α Methyl 5 HT	57	5	295/545	eluate (3 ml N HCl)
N N D methyl 5 HT (bufotenine)	44	4 5	295/545	eluate (3 ml N HCl)
5 Methoxytryptamine	46	5	295/545	eluate (3 ml N HCl)
N-Acetyl 5 HT	21	5	295/545	effluent (about 70%)
N Acetyl 5-methoxytryptamine (melatonin)	18 5	4 5	295/545	effluent (about 50%)
5 Hydroxytryptophan	69	6	295 545	effluent
α Methyl 5-hydroxytryptophan	54 5	5	295/545	effluent
5-Hydroxyindoleacetic acid	46	6	295 545	effluent

[1] Activation wavelength 295 mμ fluorescence wavelength 545 mμ

[2] Concentration 0 1 μg 1 2 ml

[3] Treated like the tissue blanks in Table II

[4] Set to 50

IV it is shown that this specific fluorescence disappears for all the substances examined when the samples are treated like a tissue blank. Therefore the peaks and the fading cannot be used as the sole specificity tests for the presence of 5-HT. Of the listed substances the acids and the N-acetylated amines some of which occur naturally in the brain are removed in the column procedure and do not appear in the eluate. The other amines including 5-HT can only be conclusively identified by other techniques, *e. g.* counter current distribution as used by Bogdanski *et al.* (1956) for the demonstration of 5-HT in the rabbit brain. The methods of Bogdanski *et al.* (1956) and of Bertler (1961) show a good agreement (Werdinius 1962). Since the extraction, the purification and the development of the fluorescence in the present method are similar to those described by Bertler (1961) our method can be considered specific for 5-HT, at least in the mammalian brain.

This work has been supported by the Swedish State Medical Research Council (14X—502—02). For a generous gift of reserpine we thank the Swedish CIBA Ltd, Stockholm. For technical assistance we are most grateful to Miss Birgitta Nilsdotter Hogberg.

References

BERTLER, Å., Effect of reserpine on the storage of catechol amines in brain and other tissues. *Acta physiol. scand.* 1961. *51*. 75—83.

BERTLER, Å., A. CARLSSON and E. ROSENGREN, A method for the fluorimetric determination of adrenaline and noradrenaline in tissues. *Acta physiol. scand.* 1958. *44*. 273—292.

BOGDANSKI, D. F., A. PLETSCHER, B. B. BRODIE and S. UDENFRIEND, Identification and assay of serotonin in brain. *J. Pharmacol.* 1956. *117*. 82—88.

GUNNE, L. M., Catecholamines and 5-hydroxytryptamine in morphine tolerance and withdrawal. *Acta physiol. scand.* 1963. *58*. Suppl. 204. 1—91.

QUAY, W. B., Differential extractions for the spectrophotofluorometric measurement of diverse 5-hydroxy- and 5-methoxyindoles. *Anal. Biochem.* 1963. *5*. 51—59.

SNYDER, S. H., J. AXELROD and M. ZWEIG, A sensitive and specific fluorescence assay for tissue serotonin. *Biochem. Pharmacol.* 1965. *14*. 831—835.

UDENFRIEND, S., D. F. BOGDANSKI and H. WEISSBACH, Fluorescence characteristics of 5-hydroxytryptamine (serotonin). *Science* 1955. *122*. 972—973.

VANABLE, Jr., J. W., A ninhydrin reaction giving a sensitive quantitative fluorescence assay for 5-hydroxytryptamine. *Anal. Biochem.* 1963. *6*. 393—403.

WERDINIUS, B., Effect of temperature on the action of reserpine. *Acta pharmacol. (Kbh.)* 1962. *19*. 43—46.

Acta physiol. scand. 1967. 69. 95—101

From the Department of Pharmacology, University of Goteborg, Sweden

Further Studies on Monoamine Metabolism and Behaviour in Rabbits Chronically Treated with Reserpine

By

JAN HÄGGENDAL, MARGIT LINDQVIST and BJÖRN-ERIK ROOS

Received 2 August 1966

Abstract

HÄGGENDAL, J, M LINDQVIST and B-E ROOS *Further studies on monoamine metabolism and behaviour in rabbits chronically treated with reserpine* Acta physiol scand 1967. *69* 95—101

[illegible]

cally treated animals the deaminated metabolites appeared essentially as after a single dose of reserpine whereas normetanephrine showed a slower recovery in the chronically treated animals Gross inspection of behaviour and sympathetic activity following DOPA injection revealed no clearcut changes in the response to DOPA in rabbits chronically treated with reserpine compared to normal animals

The findings that reserpine depresses several central nervous functions as well as peripheral symphathetic functions and causes a decrease in monoamine levels have been of great importance for the studies of the physiological role of the monoamines (for review see Carlsson 1965) Changes in the monoamine levels in brain and peripheral tissues, brought about by drugs or other procedures, have often been directly correlated to the function This has, however, been discussed and criticized by e g Everett and Wiegand (1962) and Haggendal and Lindqvist (1962, 1963)

After daily administration of small doses of reserpine to rabbits the major fraction of the monoamines in brain and peripheral tissues was removed without any marked functional disturbances about one day after the last reserpine injection The reserpine signs observed for some hours after every daily reserpine dose were temporally correlated to changes in the small remaining monoamine levels

In the present investigation we have continued the studies along the following lines

1 The monoamines were estimated in brain and heart when the first signs of reserpine action appeared after i v administration of the drug to see if functional changes could occur without any changes in the monoamine levels

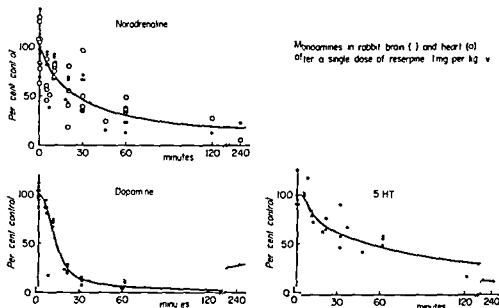


Fig 1

2 Long term treatment and single doses of reserpine were compared with respect to their influence on monoamine metabolism as reflected in metabolite levels in brain normetanephrine (NM) homovanillic acid (HVA) and 5 hydroxyindoleacetic acid (5 HIAA)

3 The effect of L DOPA administration on the gross behaviour was studied in long term reserpine treated rabbits and normal rabbits in order to see if this treat ment with reserpine caused an altered response to DOPA An increased response to DOPA might represent signs of sensitization to catecholamines

Methods

respect vely

Results

1 *Monoamine levels and symptoms after a single reserpine inject on*

Fig 1 shows the levels of NA DA and 5 HT in rabbit brain and NA in rabbit heart after a single dose of reserpine (1 mg/kg i v) The 5 HT levels in brain decreas

TABLE I Effect of reserpine on normetanephrine levels in rabbit brain (µg/g)

Hours after the last reserpine injection	Single reserpine dose		Long term reserpine treatment
	0 2 mg/kg	1 mg/kg	0 2 mg/kg daily
4	[1] 0 002	0 005	[1] 0 005
	[1] 0 002		[1] 0 000
			0 003
24 28	<0 015	0 022	0 002
		0 018	
30—52			[1] 0 001
			[1] 0 005
			[1] 0 006
			[1] 0 000
72		0 030	0 001
Untreated Mean ± s e m. 0 051 ± 0 005			

[1] Häggendal (1963 b)

ed more slowly than the catecholamine levels Within 60 min 5 HT in brain decreased to about 55 per cent and NA to about 30 per cent of the normal values The decrease of DA was more rapid than the decrease of NA Only 10 per cent of the DA remained 30 min after the injection The decrease of NA in the heart was similar to that of NA in the brain

The animals were kept and observed at room temperature Within less than 10 min the following symptoms were detectable a slight miosis, in some cases also a slight ptosis, and the muscular tone appeared to be diminished After 20 to 30 min the signs were more obvious One hour after the injection the effect of reserpine was strong sedation (decreased motor activity and lowered responsiveness to stimuli), typical posture miosis and ptosis

When the first signs of reserpine action appeared, the major part of the monoamines was still present in the brain

2 *Monoamine metabolism during reserpine treatment*

a Normetanephrine NM was estimated in whole brains of rabbits which had received a *single* dose of reserpine (0 2 or 1 0 mg/kg) or had chronically been treated with reserpine (0 2 mg/kg daily for 7 to 13 weeks) The rabbits were killed 4 hrs and about 24 hrs and 3 days after the daily administration was discontinued The values were very low and are shown in Table I

b 5 Hydroxyindoleacetic acid and homovanillic acid 5 HIAA NA and DA were estimated in rabbit brain stem and HVA, NA and DA in *corpus striatum* 4 hrs 24 hrs and 3 days after the chronical treatment with reserpine was discontinued

After 4 hrs the levels of the acids were considerably raised The concentrations remained high after 24 hrs After 3 days normal values were obtained

TABLE II Effect of reserpine on monoamines and acid metabolites in rabbit brain stem and corpus striatum Single values expressed in µg/g tissue

Brain stem	Long term treatment 0 2 mg reserpine/kg s c daily			Single dose[1] 2—5 mg reserpine/kg i v
Hours after reserpine	NA	DA	5-HIAA	5 HIAA
4	0 02	0 09	0 7	0 8
24	0 02	0 18	0 7	0 7
72	0 08	0 13	0 3	0 3
Normal	0 36	0 23	0 4	0 4
Corpus striatum	**Long term treatment 0 2 mg reserpine/kg s c daily**			**Single dose[1] 1 mg reserpine/kg i v**
Hours after reserpine	NA	DA	HVA	HVA
4	0 02	0 15	3 3	5 8
24	0 02	0 42	4 8	4 1
72	0 02	0 75	2 8	2 5
Normal	0 06	3 14	2 3	2 3

[1] Roos Andén and Werdinius (1964)

Earlier investigations have shown that after a single dose of reserpine (1, 2 or 5 mg/kg i v) to rabbits the levels of 5 HIAA and HVA increased (Roos, Anden and Werdinius, 1964) The present results showed that the metabolite levels were high and that the pattern seemed to be the same after long term treatment with small daily doses of reserpine as after a single dose (see Table II)

3 *Effect of L-DOPA on the behaviour*

L-DOPA, 10, 20 and 100 mg per kg, was given i v to 3 normal rabbits and to 3 long term reserpine treated rabbits 24 hrs after the last reserpine injection Signs of dopa effect piloerection pupil dilatation, increased breathing rate and kick movements with the hind limbs were observed in both groups, with the strongest signs after the highest dopa dose No difference could be detected between the groups

Discussion

In many respects the central and peripheral catecholaminergic neurons are similar Histochemically they have in principle the same appearance and after administration of many drugs they behave similarly The rate of disappearance of NA was about the same in rabbit brain as in heart after a single injection of reserpine The speed of the recovery of NA after a single reserpine injection was also about the same for brain and heart (Haggendal and Lindqvist 1964)

After long term treatment with reserpine rabbits were found to be almost normal about one day after the last injection with respect to gross behaviour in spite of very

low levels of monoamines in brain, heart and adrenals (Haggendal and Lindqvist 1963) Thus a large fraction of the monoamines in the tissues, central or peripheral, could be removed without any marked functional disturbances The signs of reserpine action observed for some hours after the daily reserpine injection could be temporally correlated to changes in the small remaining monoamine fractions in the brain (Haggendal and Lindqvist 1964)

With other technique the different monoamine fractions and their functions have been studied in peripheral tissues and the active fraction was found to be very small (Anden and Magnusson 1964, Sedvall 1965, Malmfors 1965) which is in good agreement with our results

1 Most of the monoamines were still present in the brain when the typical reserpine signs first appeared after a single dose of the drug (Fig 1) This is in agreement with the view that the monoamines are present in two main fractions, one small fraction correlated to the behaviour and one large fraction, probably a storage pool The small fraction seems to be less than 10 per cent of the total amount, and thus quantitative changes in this pool are hard to observe when the larger fraction is present When the first reserpine signs were observed the small fraction might thus have been proportionally more reduced than the total amount of monoamines in the brain It seems that after reserpine the large fraction cannot rapidly enough be transferred to the small functionally important fraction and be utilized The smaller fraction was thus depleted of transmitter more rapidly than the larger pool This is in agreement with the results on skeletal muscular tissue (Sedvall 1965), but not with those on heart auricle (Gaffney, Chidsey and Braunwald 1963) and iris (Malmfors 1965) There may be differences between different tissues and species The technique used for the investigations may also be of importance

2 The amount of NM formed by 3 0 methylation of NA in the extraneuronal space depends on *i a* the amount of NA available for the catechol 0 methyl transferase

Four hrs after a *single* dose of reserpine, 0 2 or 1 0 mg/kg s c , very low levels of NM were obtained At the same time the reserpine signs were evident Within 24 to 72 hrs, when the reserpine signs almost had disappeared the NA and NM still were low but had increased somewhat After reserpine a large amount of NA, obviously not necessary for the function, had been removed Since the NM levels still were low it seems possible that the "lacking NM was related to this removed NA

After *long term* treatment with reserpine the NM levels were very low 4 hrs and also 72 hrs after the last injection

The NM pattern thus differed somewhat after 3 days in acute and chronically reserpine treated animals The explanation to this is probably complicated and the results may more be due to the amount of reserpine used than to the reiteration of the reserpine injections

With respect to the acid metabolites 5 HIAA and HVA the acute and chronically reserpine treated animals were similar

According to these results the NA metabolism was principally the same whether

the animals had received a single injection or had been long term treated with reserpine The NM levels however, differed somewhat

3 After reserpine treatment an increased sensitivity to injected NA has been found Fleming and Trendelenburg (1961) demonstrated this on the nictitating membrane after daily reserpine injections for at least 7 days but not after a single reserpine dose For studies on the blood pressure see *e g* Zwieten *et al* (1965)

In this investigation the long term reserpine treated animals did not show any marked differences in their responses to DOPA administration compared to the responses in not reserpine treated animals When the daily administration of reser pine was discontinued the levels of the monoamines increased at the same rate as after a single dose of reserpine and without any signs of excitation according to our earlier study (Haggendal and Lindqvist 1964) Furthermore, the metabolism of the monoamines after long term treatment reflected in the levels of 5 HIAA and HVA appeared to be about the same as after a single injection For NM there was some differences, however But the results appear by the gross to indicate that the mono-amine synthesis and metabolism had not changed during the long term treatment and may also indicate that sensitization to the transmitter not had occurred to any marked extent

The reason why these results differed from those of other investigators may be *e g* differences in species and technique Markiewicz (1963) found in mice at least a partial recovery from the reserpine action regarding weight and behaviour during long term treatment with reserpine In the latter part of the period of long term reserpine treatment the catecholamines in the mouse adrenals increased markedly and adrenaline returned almost to the normal level Other factors of importance may be the dose of reserpine used and also the manner of observation In this study only the gross behaviour was observed With a more sensitive technique for the observation of the behaviour and of the peripheral sympathetic effects some dis crepances might have been found When reserpine was given to rabbits in a daily dose of 0 1 mg/kg the reserpine signs after every injection seemed to decline when the animals had been treated for about 1 week (Haggendal and Lindqvist 1963) When the animals received 0 2 mg reserpine per kg and day somewhat more clear cut signs of reserpine action was found after the daily injections during the first week than later during the long term treatment This adaptation might be due to sensitiza tion of the receptors towards the monoamines but other explanations are also possible

These studies have given further evidence to the view that the function is closely correlated to a small fraction of the transmitter and that this fraction can be disclosed by long term treatment with reserpine without any pronounced signs of changed monoamine metabolism or marked signs of sensitization

Recently this small fraction has been discussed consisting of new synthetized amine storage granules formed in the cellbodies and rapidly transported down the axons to the nerve terminals (Dahlstrom and Haggendal 1966)

This research was supported by the Swedish State Medical Research Council (B 67-14X 166-03), (14X-165 02) and Air Force Office of Aerospace Research under Grant AF EOAR 65 56 through the European Office of Aerospace Research OAR, United States Air Force
For generous supplies of Serpasil we are indebted to the Swedish Ciba Ltd , Stockholm

References

ANDÉN, N -E and T. MAGNUSSON, Functional significance of noradrenaline depletion by alpha [illegible]

[illegible]

CARLSSON, A and M LINDQVIST, In-vivo decarboxylation of α methyl DOPA and α methyl metatyrosine *Acta physioll scand* 1962 *54* 87—94
CARLSSON A and B WALDECK, A fluorimetric method for the determination of dopamine (3-hydroxytyramine) *Acta physiol scand* 1958 *44* 293—298
DAHLSTRÖM, A and J HÄGGENDAL, Recovery of noradrenaline levels after reserpine as compared to the life span of amine storage granules in rat and rabbit *J Pharm Pharmacol* 1966 *18* 750—782
EVERETT, G M and R G WIEGAND, Central amines and behavioral states A critique and new [illegible]

[illegible] 1963 *12* 264—268
HÄGGENDAL, J , An improved method for fluorimetric determination of small amounts of adrenaline [illegible]

[illegible]

MALMFORS, T , Studies on adrenergic nerves *Acta physiol scand* 1965 *64* Suppl 248
MARKIEWICZ, L , Biochemical and functional effects of long-term administration of reserpine in mice *Acta physiol scand* 1963 *58* 376—380
ROOS B E On the occurrence and distribution of 5-hydroxyindoleacetic acid in brain *Life Sci* 1962 *1* 25—27
ROOS, B E N -E ANDÉN and B WERDINIUS, Effect of drugs on the levels of indole and phenolic acids in the central nervous system *Int J Neuropharmacol* 1964 *3* 117—122
SEDVALL, G and J THORSON, Adrenergic transmission at vasoconstrictor nerve terminals partially depleted of noradrenaline *Acta physiol scand* 1965 *64* 251—258
VAN ZWIETEN P A S WIDHOLM and G HERTTING, Influence of cocaine and of pretreatment with reserpine on the pressor effect and the tissue uptake of injected dl-catecholamines 2 H[3] *J Pharmacol* 1965 *149* 50—56

Acta physiol. scand. 1967. 69. 102—110

From the Institute for Medical Biochemistry and the Institute of Physiology, University of Oslo Norway

Effects of Adrenaline on Coronary Flow in Isolated Perfused Rat Hearts

By

ANDERS GLOMSTEIN, ANTON HAUGE, IVAR OYE and DEREK SINCLAIR

Received 16 August 1966

Abstract

GLOMSTEIN, A, A HAUGE, I OYE and D SINCLAIR *Effects of adrenaline on coronary flow in isolated perfused rat hearts* Acta physiol scand 1967 69 102—110

The mechanism for adrenergic regulation of coronary flow has been studied in an isolated, working rat heart preparation It is concluded that adrenaline affects coronary flow in this preparation through three separate modes of action 1 Constriction of the coronary resistance vessels mediated through α type of adrenergic receptos in these vessels 2 Dilatation of the coronary resistance vessels mediated through β type of adrenergic receptors in these vessels 3 Dilatation of the coronary resistance vessels as a consequence of increased myocardial metabolism after adrenaline

Adrenaline is thought to affect coronary flow rate in two basically different ways One is a direct adrenergic effect on the tone of the coronary vessels, the other is an indirect effect secondary to the change in the physical and metabolic activity of the muscle brought about by adrenergic stimulation Early work on regulation of coronary flow has been reviewed by Anrep (1926) who stated that the usual result of adrenergic stimulation is an increase in coronary flow However, Brodie and Cullis (1911) found that small doses of adrenaline diminished coronary flow and suggested that the primary effect of adrenaline may be a coronary vaso-constriction which is followed by a vasodilatation as the metabolic effects of adrenergic stimulation become apparent This is in agreement with more recent studies (Katz *et al* 1938, Berne 1958, Gregg and Fisher 1963), but the primary coronary constrictor effect is still not universally accepted (Hirche 1966)

In the present investigation the adrenergic control of coronary flow rate has been studied in an isolated working rat heart preparation The data lead to the conclusion that the direct effect of adrenaline on coronary vessels involves vasoconstriction mediated through α-type of adrenergic receptors as well as vasodilatation mediated through β-type receptors In addition to these two primary and antagonistic actions, adrenaline indirectly causes coronary vasodilation as a consequence of its effects on the myocardium Metabolic rather than physical factors were found to be responsible for the secondary raise in coronary flow after adrenaline

Methods

Female breeder rats (250—300 g) of local strain (originally Wistar) were used throughout The

Results

Adjustment of coronary flow to cardiac activity

Adjustment of coronary flow to the physical activity of the isolated heart was observed when flow direction of the perfusate was changed from anterograde (working hearts) to retrograde (hearts contracting without doing any pump work) The coronary flow rate always fell when the hearts ceased to pump If the hearts again were allowed to resume anterograde pumping activity, coronary flow immediately rose to the initial level The oxygen tension in the coronary outflow perfusate remained relatively constant when the flow direction was changed, and the difference in cardiac oxygen consumption reported in a previous paper (Hauge and Oye 1966) was a result of changes in coronary flow rather than in oxygen extraction A further decrease in coronary flow occurred when the hearts were made asystolic by increasing the potassium concentration of the perfusate However, the fall in coronary flow did not coincide with the cessation of contractions In fact, an increased flow was occasionally observed during the initial phase of asystolia, followed by a rapid decrease in the course of 1—2 min These findings show that a mechanism for regulation of coronary flow to meet the requirements for oxygen during different work loads persisted in the isolated perfused heart preparation The findings also indicate that the relationship between physical activity and coronary flow was an indirect one, probably mediated through metabolic factors rather than through physical factors alone

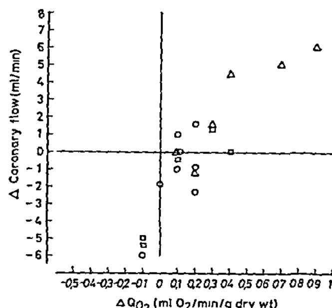


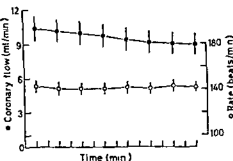
Fig 1 Change in coronary flow following adrenaline administration to hearts arrested with potassium plotted against the changes in myocardial oxygen consumption The values are taken during the period 3—5 min after the addition of adrenaline The doses of adrenaline used were 0 25 µg/ml (□), 0 75 µg/ml ( ) and 2.5 µg/ml (△)

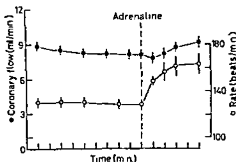
The effect of adrenaline on coronary flow in working and arrested hearts

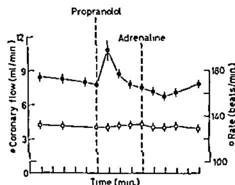
Adrenaline increased the rate of contractions (chronotropic effect) and the maximum systolic pulse pressure (referred to here as inotropic effect) In working hearts adrenaline increased the oxygen consumption as well as coronary flow when measured 3—5 min after adrenaline administration This increase in flow might be due to direct actions of adrenaline on the coronary vascular bed or might be secondary to changes in the physical and metabolic activity of the muscle

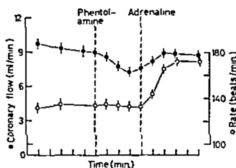
In hearts arrested with high potassium concentration in the perfusate, as described under "Methods the effect of adrenaline on coronary flow could be studied in the absence of inotropic and chronotropic effects In order to demostrate a possible relationship between effect on coronary flow and metabolic effects of adrenaline in the arrested hearts, changes in flow and changes in oxygen consumption were plotted in a common coordinate system (Fig 1) An increase in oxygen consumption of 0 2 ml min g dry muscle or more, was associated with increased coronary flow A reduction in coronary flow was found in hearts where the effect on oxygen consumption was small or absent

In working hearts coronary flow was usually found to be increased 3—5 min after adrenaline However, the immediate effect was often a reduction in flow In working hearts this occurred despite an early inotropic and chronotropic effect The early effect of adrenaline on coronary flow was examined more closely in a series of 32 expts (Fig 2) As seen in Fig 2 a, cardiac rate remained constant in the control group of hearts whereas there is a steady fall in coronary flow rate of about 0 1 ml per minute This fall was corrected for when changes in coronary flow were calculated in the other experimental groups Fig 2 b shows the effect of adrenaline









[illegible] (●) [illegible] (○) [illegible]

beginning of perfusion

A Controls perfused without adrenaline addition

B Adrenaline 2 5 μg/ml added

C Propranolol 2 5 μg/ml added followed in 4 min by the addition of adrenaline to the same concentration

D Phentolamine 2 5 μg/ml added followed in 4 min by the addition of adrenaline to the same concentration

on coronary flow and cardiac rate The increase in flow develops later and more gradually than the chronotropic effect, and appears to be preceded by a slight decrease in flow

Modification of the effect of adrenaline on coronary flow by adrenergic blocking agents

In an attempt to analyze further the effect of adrenaline on coronary flow, adrenergic blocking agents were used The β-adrenergic receptor blocking agent propranolol (Fig 2 c) and the α-adrenergic receptor blocking agent phentolamine (Fig 2 d) both affected coronary flow and also modified the effect of a subsequent dose of adrenaline Propranolol by itself caused a transient and rapid increase in coronary flow whereas phentolamine caused a more gradual reduction Cardiac output

(which is not plotted in the diagram) showed a slight decrease following both blocking agents As expected, the chronotropic effect of adrenaline was completely blocked by propranolol, but not by phentolamine A rapid increase in flow followed adrenaline administration to the phentolamine treated hearts, whereas flow rate continued to fall in the propranolol treated group In both cases the late effect of adrenaline was an increase in flow In the propranolol group this increase was associated with a delayed inotropic response The rise in coronary flow after propranolol and the fall after phentolamine was found to be statistically significant ($p < 0.005$) However, the fall after adrenaline seen in Fig 2 c and the rise after adrenaline seen in Fig 2 d could be interpreted as rebound effects in the *recovery from the previous* propranolol or phentolamine actions In order to examine this problem further a new series of 16 experiments were performed In this group adrenaline was not added until 10 min after addition of propranolol or phentolamine (Fig 3) This allowed the *flow to stabilize before the effect of adrenaline was tested* Adrenaline caused a marked

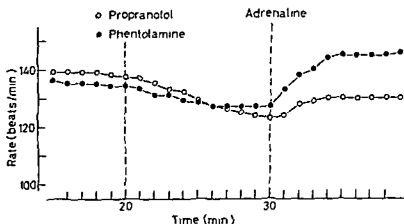


Fig 3 A

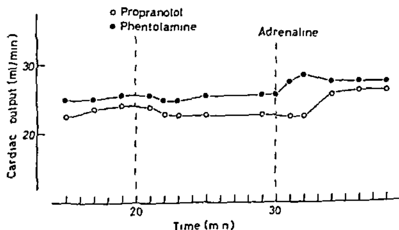


Fig 3 B

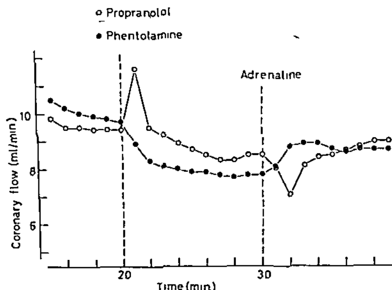


Fig 3 C

Fig 3 The effect of adrenaline (2 5 μg/ml) added to the perfusate 10 min after the addition of propranolol ( ) or phentolamine (●) Both blocking agents were added to the concentration of 2 5 μg/ml The experiments were performed alternately and all measurements taken after the hearts appeared to have reached a steady state The diagrams show mean values of 8 experiments in each group A Cardiac rate B Coronary flow C Cardiac output

chronotropic effect in the phentolamine group and a slow rise in the heart rate could be observed in the propranolol-treated preparations (Fig 3 a) The left ventricular output (Fig 3 b) fell slightly after the addition of both blocking agents In both groups a marked increase in cardiac output followed adrenaline administration, but in the propranolol group this response was delayed for about 2 min The coronary flow rate (Fig 3 c) showed the usual rapid rise after the addition of propranolol, and the usual fall after the addition of phentolamine The flow then reached a steady level after about 7 min In the propranolol-treated hearts adrenaline caused a rapid fall in coronary flow from 8 5 ± 0 7 ml/min (8 hearts) to a minimum of 7 1 ± 0 5 ml min in 2 min This fall was significant (0 < 0 005) The flow then climbed slowly to rise above the pre-addition level This late rise in coronary flow which coincided with the rise in cardiac rate and ventricular output, was interpreted as due to adrenaline overcoming the β blocking agent In the phentolamine-treated hearts adrenaline caused a rapid rise in coronary flow from 7 7 ± 0 7 ml/min (8 hearts) to 8 9 ± 0 7 ml/min 3 min after the addition (p < 0 001) and the flow then stabilized at a level slightly above the controls expected

The decrease in flow after adrenaline in the propranolol group is clearly dissociated from the inotropic and chronotropic effects which were delayed by about 2 min The increase in flow after adrenaline in the phentolamine group coincided with the increased physical activity of the muscle, but appeared earlier than the increase

observed after adrenaline in the absence of blocking agents This early increase in flow did not depend on the chronotropic or inotropic effect and could also be observed during experimental asystolia In a series of 4 hearts arrested by increasing the potassium concentration of the perfusate, the coronary flow rate was found to be 3 2 ± 0 3 ml/min 5 min after phentolamine, and increased promptly to a maximum of 5 8 ± 0 4 ml/min on adrenaline administration The initial decrease in flow after adrenaline in propranolol treated hearts was also found to be present during experimental asystolia These early effects on coronary flow were therefore not secondary to changes in the contractile activity of the heart, but reflected direct actions of adrenaline on the coronary vascular bed

Discussion

In the experiments presented above, adrenaline was found to affect coronary flow through more than one mechanism and the effects observed can be classified as follows

1 An increase in coronary flow, associated with increased oxygen consumption, appearing 2—3 min after adrenaline administration This increase was most prominent in working hearts in which adrenaline increased the rate and force of contraction, but also appeared during experimental asystolia In asystolic hearts this increase was only seen when adrenaline increased myocardial oxygen consumption

2 An early slight decrease in flow was usually observed to precede the increase in flow described above Following β blockade this initial effect was potentiated and found to be highly significant The effect could be dissociated from the chronotropic and inotropic effects and also appeared in hearts arrested with high concentration of potassium in the perfusate

3 An early increase in flow rather than a decrease as described above, was occasionally observed after adrenaline Following α blockade the early effect of adrenaline was always found to be an increase in flow, and this effect was now statistically significant In the working hearts this early effect coincided with the chronotropic and inotropic effects, but it could be dissociated from any changes in physical activity by using hearts arrested with high potassium concentrations in the perfusate

The early changes in flow most likely reflect the direct action of adrenaline on the coronary vascular bed The presence of primary adrenergic coronary vasoconstriction has been observed by several previous workers (Brodie and Cullis 1911, Green *et al* 1942 Scherlis and Provenza 1958, Hardin *et al* 1961) Recently evidence for a primary adrenergic vasodilator mechanism in the dog heart has been presented by Klocke *et al* (1965) and by Hirche (1966) The presence of both α receptors (constrictor) and β receptors (dilator) has been proposed by Doutheil *et al* (1964) and by Takenata (1966), whereas Mendez and Kabela (1966) suggested that coronary vasoconstriction is achieved by stimulation of coronary β receptors

Our findings indicate that both α receptor and β receptors are involved in the direct adrenergic regulation of coronary vascular tone in the rat heart, stimulation

of the α receptors causing constriction and stimulation of the β receptors causing dilatation Parrat and Grayson (1966) observed that administration of propranolol *in vivo* caused a decrease in coronary flow, probably due to abolition of sympathetic vasodilator tone and unmasking of a vasoconstrictor action of adrenaline In the isolated heart of our experiments where normal sympathetic tone is absent, propranolol caused a rapid, transient increase in flow Most adrenergic blocking agents show some sympaticomimetic activity which is believed to reflect the interaction of the blocking agent with the receptor involved The rapid increase in flow after addition of propranolol to the isolated heart may thus be additional evidence for the presence of β receptors in the coronary resistance vessels, and the decrease in flow observed after addition of phentolamine is similarly evidence for the presence of α receptors in these vessels The presence of both types of receptors in the coronary vessels is also supported by the finding that in the absence of added blocking agents the early effect of adrenaline could be either a transient decrease or an increase in flow, although a decrease occurred in most of the hearts studied

The increase in coronary flow which developed 2—3 min after adrenaline administration appeared to be independent of the initial effect of adrenaline and was closely associated with the increase in oxygen consumption as discussed in a previous paper (Hauge and Oye 1966) This effect on coronary flow is interpreted as secondary to the metabolic effects of adrenaline The many lines of evidence for a close association between cardiac metabolism and coronary flow have been discussed in a recent review by Berne (1964) Oxygen consumption is, at least in part, controlled by the rate of ATP utilization and the hypothesis that a degradation product of ATP, adenosine, is an important regulator of coronary flow rate in vivo (Berne 1963) is particularly attractive, because this would mean that oxygen consumption and coronary flow are coupled through one basic mechanism of regulation This mechanism of autoregulation offers an explanation for the adjustment of coronary flow to the oxygen consumption in the isolated heart during various work loads, and could also account for the secondary increase in coronary flow after adrenaline in the isolated rat heart during work as well as in the asystolic state

This work was supported by a grant from Nasjonalforeningen Det Norske Råd for Hjerte og Karsykdommer

References

ANREP G V The regulation of coronary circulation *Physiol Rev* 1926 *6* 596—679

BERNE R M Cardiac nucleotides in hypoxia possible role in regulation of coronary blood flow *Amer J Physiol* 1963 *204* 317—326

BERNE R M Effect of epinephrine and norepinephrine on coronary circulation *Circulat Res* 1958 *6* 644—652

BERNE R M Regulation of coronary blood flow *Physiol Rev* 1964 *44* 1—29

BRODIE T G and W C CULLIS The innervation of the coronary vessels *J Physiol* (Lond) 1911 *43* 313 324

DOUTHEIL, U H G BRUGGENCATE and K KRAMER Coronarvasomotorik unter L-noradrenalin und isopropylnoradrenalin nach blockierung der adrenergischen β-receotoren durch Nethal de *Pflügers Arch ges Physiol* 1964 *281* 181—190

GREEN H D R WEGRIA and H H BOYER. The effect of epinephrine and pitressin on the coronary artery inflow in anesthetized dogs *J Pharmacol exp Therap* 1942 *76* 3 8—386

observed after adrenaline in the absence of blocking agents This early increase in flow did not depend on the chronotropic or inotropic effect and could also be observed during experimental asystolia In a series of 4 hearts arrested by increasing the potassium concentration of the perfusate, the coronary flow rate was found to be 3 2 $\pm$ 0 3 ml/min 5 min after phentolamine, and increased promptly to a maximum of 5 8 $\pm$ 0 4 ml/min on adrenaline administration The initial decrease in flow after adrenaline in propranolol treated hearts was also found to be present during experimental asystolia These early effects on coronary flow were therefore not secondary to changes in the contractile activity of the heart, but reflected direct actions of adrenaline on the coronary vascular bed

Discussion

In the experiments presented above, adrenaline was found to affect coronary flow through more than one mechanism and the effects observed can be classified as follows

1 An increase in coronary flow, associated with increased oxygen consumption, appearing 2—3 min after adrenaline administration This increase was most prominent in working hearts in which adrenaline increased the rate and force of contraction, but also appeared during experimental asystolia In asystolic hearts this increase was only seen when adrenaline increased myocardial oxygen consumption

2 An early slight decrease in flow was usually observed to precede the increase in flow described above Following β blockade this initial effect was potentiated and found to be highly significant The effect could be dissociated from the chronotropic and inotropic effects and also appeared in hearts arrested with high concentration of potassium in the perfusate

3 An early increase in flow rather than a decrease as described above, was occasionally observed after adrenaline Following α blockade the early effect of adrenaline was always found to be an increase in flow and this effect was now statistically significant In the working hearts this early effect coincided with the chronotropic and inotropic effects but it could be dissociated from any changes in physical activity by using hearts arrested with high potassium concentrations in the perfusate

The early changes in flow most likely reflect the direct action of adrenaline on the coronary vascular bed The presence of primary adrenergic coronary vasoconstriction has been observed by several previous workers (Brodie and Cullis 1911, Green *et al* 1942 Scherlis and Provenza 1958, Hardin *et al* 1961) Recently evidence for a primary adrenergic vasodilator mechanism in the dog heart has been presented by Klocke *et al* (1965) and by Hirche (1966) The presence of both α receptors (constrictor) and β receptors (dilator) has been proposed by Doutheil *et al* (1964) and by Takenata (1966) whereas Mendez and Kabela (1966) suggested that coronary vasoconstriction is achieved by stimulation of coronary β receptors

Our findings indicate that both α receptor and β receptors are involved in the direct adrenergic regulation of coronary vascular tone in the rat heart, stimulation

Acta physiol. scand. 1967. 69. 111—118

From the Departments of Physiology and of Clinical Chemistry, Veterinarhögskolan, and the Department of Endocrinology, Karolinska Sjukhuset, Stockholm, Sweden

Studies of the Importance of the Thyroid and the Sympathetic System in the Defence to Cold of the Goat

By

B ANDERSSON, L EKMAN, B HÖKFELT[1], M JOBIN[2], K OLSSON and D ROBERTSHAW[3]

Received 6 September 1966

Abstract

ANDERSSON, B, L EKMAN, B HOKFELT, M JOBIN, K OLSSON and D ROBERTSHAW *Studies of the importance of the thyroid and the sympathetic system in the defence to cold of the goat* Acta physiol scand 1967 *69* 111—118

[illegible] accentuated, and the increase in urinary excretion of catecholamines (especially of adrenaline) was generally much greater than that of the euthyroid goat In 3 out of 4 hypothyroid goats studied the catecholam[illegible] was also higher than that found in euthy[illegible] of the hypothyroid goats rose by about 3[illegible] in the euthyroid animals It is concluded t[illegible] hypothyroid goats have to compensate [illegible] in adrenaline secretion Bilateral splanch[illegible] complete absence of adrenaline in the u[illegible] and the shivering seen during local cool[illegible] glycemic response to hypothalamic cooli[illegible]

Adrenaline, noradrenaline and thyroid hormones seem to be of major importance in cold-stimulated nonshivering thermogenesis, and a synergism apparently exists between these hormones so that the calorigenic action of the catecholamines is potentiated by thyroxine (cf Carlson 1960) Temperature sensitive neurons in the thermoregulatory centre in the anterior hypothalamus participate in the control of these hormonal cold defence mechanisms Local warming of this "centre" inhibits the activation of the sympathetic system and the thyroid activation normally occurring during a general cold stress Local cooling of the "centre", on the other

[1] Present address Dept of Endocrinology Allmanna Sjukhuset, Malmo, Sweden
[2] Postdoctoral Research Fellow N R C Canada From the Dept of Physiol Medical School, Laval University Quebec P Q
[3] On leave from the Dept of Physiol The Hannah Dairy Research Institute, Ayr Scotland

hand, causes increased secretion of catecholamines and of thyroid hormone (*cf* Andersson, Gale and Hökfelt 1964 a).

The present series of experiments was performed in order to study how removal of the thyroid or splanchnicotomy would change the response of goats to cold exposure and to local cooling of the hypothalamic thermoregulatory "centre".

Methods

Five adult female goats (in the follo[illegible]
normal goats were used as cont[illegible]
37 to 43 kg Three of the goats [illegible]
implanted into the anterior hyp[illegible]
for local cooling of the anterior [illegible]

Surgical thyroidectomy Surgical removal of the thyroid gland was performed under general anesthesia in goats I, II, III and IV.

Radiothyroidectomy In order to destroy and to prevent the further development of ectopic thyroid present in the animals, the surgically thyroidectomized goats were given large doses (20 or 25 mCi) of I[131] two to four times during one to two months following the operation For further details on the radiothyroidectomy and on radioisotope scanning (performed to detect ectopic thyroid tissue), see Ekman (1965)

[illegible]

of the anter[illegible]

[illegible] blood glucose was determined enzymatically as described by Levin and Linde (1962)

Supplementary treatment For certain periods the thyroidectomized goats were given intravenously Thyroxine "Roche" in doses of 0 25 or 0 5 mg per day

Results

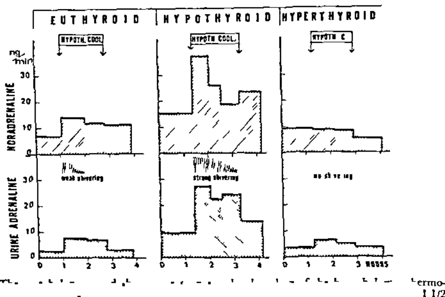
A *Effects of thyroidectomy*

1) General effects

The "thermode" goats I and II were studied for 5 and 7 months respectively after surgical thyroidectomy The main changes in general appearance that developed in these goats were a marked increase in body weight (10—15 per cent), an increase in fur growth and a certain degree of sluggishness The same symptoms developed during the first 6 months after thyroidectomy in goats III and IV, followed, at a later stage by marked thickening and softening of the skin, local loss of hair, symptoms of laminitis and the development of myxoedema The serum PBI, being within the range of 4 5 to 5 5 μg/100 ml before thyroidectomy, had in all animals dropped to levels between 0 1 and 0 4 μg/100 ml 3 months after the operation.

2) *Effects on urinary excretion of catecholamines and on the response to cold a) Basic catecholamine excretion* The urinary excretion of adrenaline and of noradrenaline at a

1 Grateful acknowledgement is made to Dr G Widstrom, M D, Head of the Clinical Lab, Danderyds Sjukhus, Danderyd, by whose courtesy the serum PBI determinations were made



[illegible] ermo-
1 1/2

room temperature of $20 \pm 2°$ C was determined during 1 to 3 hr periods on 3 occasions in each of the "thermode" goats I and II 1 1/2 to 2 months after thyroidectomy These determinations were made on urine collected during control periods before the application of hypothalamic cooling Comparison was made with excretion rates during similar periods of urine collection in the same animals before thyroidectomy An approximately 100 % increase in both adrenaline and noradrenaline excretion was observed in goat I during the hypothyroid state (serum PBI 0 2 μg/100 ml) whereas the catecholamine excretion of goat II (serum PBI 0 4 μg/100 ml) was not significantly raised as compared to preoperative level

The catecholamine excretion at room temperature was also followed in goats III and IV in 4 expts performed 20 and 21 months after thyroidectomy Their serum PBI at this stage was 0 2 μg/100 ml Since no determinations of catecholamine excretion had been made before thyroidectomy in goats III and IV, the excretion rates were compared to those found in parallel experiments made in 2 control goats and to results obtained in earlier studies of the catecholamine excretion of euthyroid goats (Andersson *et al* 1963 b) The urinary excretion of adrenaline and of noradrenaline (calculated for 40 kg b wt) was about 100 per cent higher in the hypothyroid goats than in the controls and in euthyroid goats studied earlier (Fig 2)

b) Response to local cooling of the anterior hypothalamus The response to 2 hr periods of local cooling of the anterior hypothalamus at room temperature was studied on several occasions in goats I and II during the second month after thyroidectomy At this stage the serum PBI of goat I had dropped to 0 2 μg/100 ml and that of

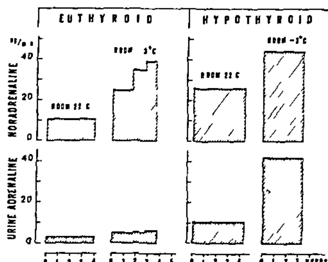


Fig 2 The catecholamine excretion at room temperature and during acute cold exposure in an euthyroid control goat (*left*) and in goat III 20 months after surgical thyroidectomy followed by radiothyroidectomy (*right*)

The catecholamine excretion is calculated for 40 kg b wt in both animals

goat II to 0 4 μg/100 ml Like before thyroidectomy, both goats reacted with a 2° C rise in rectal temperature during the period of central cooling However, the shivering response to hypothalamic cooling had become much greater after thyroidectomy in both animals Also the catecholamine response to local cooling of the anterior hypothalamus was markedly potentiated after thyroidectomy in goat I (Fig 1) During central cooling before thyroidectomy in this animal the peak excretion rate (calculated per 40 kg b wt) was 16 ng/min for noradrenaline and 7 5 ng/min for adrenaline Two months after thyroidectomy it was 37 ng/min for noradrenaline and 27 ng/min for adrenaline A slight, but much less evident increase in the catecholamine response to central cooling was observed after thyroidectomy in goat II

During the 3rd and the beginning of the 4th postoperative month goat I received daily injections of thyroxine At the end of this supplementation period the catecholamine response to local cooling of the anterior hypothalamus was negligible and no shivering was observed (Fig 1, right) The serum PBI of the goat was then *10 μg/100 ml*

c) Response to external cold The response of goats III and IV to external cold (— 3° C) was tested repeatedly 6 and 20 months after thyroidectomy and was compared to that of two euthyroid control goats The urinary catecholamine excretion was followed during the latter test period The cold exposure lasted for 3 to 4 hrs Like the euthyroid controls the hypothyroid goats (serum PBI 0 2 μg/100 ml) maintained normal rectal temperature during the cold exposure This was however accomplished at the expense of very intense shivering The hypothyroid goats started to shiver strongly within 3 to 5 min after they were placed in the cold, and intense shivering continued throughout the entire cold exposure period The blood glucose of goats III and IV rose from a basic level of 40—50 mg/100 ml to 150—180 mg/

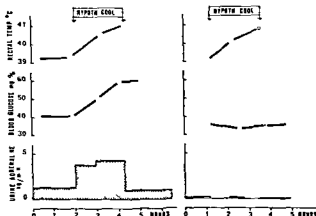


Fig 3 The response to local cooling of the thermoregulatory centre' in the euthyroid goat V before (*left*) and after splanchnicotomy (*right*)
The lack of adrenaline secretion after splanchnicotomy prevented the rise in blood glucose level but not the rise in body temperature during local cooling of the anterior hypothalamus
Room temperature in both experiments = 19 C

100 ml in the cold In contrast, the euthyroid controls did not start to shiver until half to one hr after the beginning of cold exposure and their blood glucose was only slightly elevated (10 to 20 per cent increase)

In the euthyroid controls the cold exposure gradually caused a 300 per cent increase in noradrenaline excretion whereas the adrenaline excretion was only slightly elevated (Fig 2 left) The noradrenaline excretion of the hypothyroid goats was not much higher than that of the controls in the cold, showing only about 50 per cent increase over the already elevated room temperature level (Fig 2, right, above) In contrast, the adrenaline excretion of the hypothyroid goats immediately rose in the cold to very high levels (about 40 ng/min, calculated per 40 kg b wt), showing an increase over room temperature excretion rate by about 300 per cent (Fig 2, right, below)

During the 7th postoperative month goat III received supplementary treatment with thyroxine At the end of this month the animal was exposed to cold for a 3 hr period The reaction of the goat was under these circumstances similar to that of a normal animal, i e moderate, delayed shivering and a 10 per cent increase in blood glucose only

B *Effects of splanchnicotomy*

The response to 2 hr periods of hypothalamic cooling was studied in the euthyroid goat V on 2 occasions during the 2nd week after bilateral denervation of the adrenal medulla A 2 C rise in rectal temperature occurred both before and after the operation and was accompanied by shivering However the 50 per cent rise in blood glucose observed during preoperative central cooling was no longer present after splanchnicotomy, and the urinary adrenaline excretion, which had increased by about 150 per cent during preoperative central cooling, was practically *nil* (Fig 3) The noradrenaline response to central cooling remained of the same order as that observed before splanchnicotomy

Discussion

Cold-stimulated increase in heat production without detectable muscular activity is usually named nonshivering thermogenesis Although the existence of this form of "chemical" temperature regulation has been much debated, more recent experiments have definitely shown its presence in certain mammalian species (*cf* Hart 1963) The studies of Carlson *et al* (*cf* Carlson 1960) have demonstrated the importance of the catecholamines in nonshivering thermogenesis of cold acclimated rats During cold-acclimation the calorigenic effect of adrenaline and especially of noradrenaline is potentiated in this species An increased calorigenic effect of adrenaline has also been found in association with the increased thyroid secretion in the cold (Ring 1942), and it has been shown that cold-induced nonshivering thermogenesis decreases with time following thyroidectomy (Hsieh and Carlson 1957) It thus seems that the complex phenomenon of metabolic adaptation to cold is at least in part the result of an interaction between thyroid hormone and the hormones of the sympathetic nervous system This may explain certain gradually *developing effects of repetitive local cooling of the hypothalamic thermoregulatory* "centre" of goats (*cf* Andersson *et al* 1964 a) During consecutive experimental periods involving repeated cooling of the anterior hypothalamus, the basal thyroid activity of the animals increased At the same time the catecholamine response and the shivering response to hypothalamic cooling gradually diminished, although the rise in body temperature of the animals during central cooling remained of the same order as that observed initially The repeated lowering of the temperature of the thermoregulatory "centre' had apparently increased the ability of the animals to respond with nonshivering thermogenesis, i e it had increased their acclimation to cold

The present series of experiments demonstrate that a lack of thyroid hormone has the reverse effect in the goat Thyroidectomy was found to potentiate markedly the shivering response to hypothalamic cooling in both goats studied In goat I (having the lowest serum PBI value) both the basic catecholamine excretion and that observed during hypothalamic cooling was much higher than in the same animal before thyroidectomy The reason why the catecholamine excretion of goat II (having a higher serum PBI value) did not show the same marked elevation after thyroidectomy may be that secretion of hormone from ectopic thyroid tissue was in this animal still of a magnitude sufficient to potentiate significantly the calorigenic action of the catecholamines It has been shown (Sellers and You 1950) that in the already cold-acclimated rat only very small amounts of thyroxine are required to maintain body temperature in the cold

The effects of cold exposure in the hypothyroid goats III and IV indicated an even more complete loss of the metabolic adaptation to cold Leduc's (1961) studies of catecholamine excretion in rats have shown that the immediate response to moderate cold exposure is an increased release of noradrenaline from the adrenergic nerve endings An accelerated release of adrenaline from the adrenal medulla appears later, as a secondary response during prolonged cold exposure, or may be

een earlier during a more profound cold stress The studies of the catecholamine excretion during cold exposure of the control goats show that the situation is the same in the euthyroid goat (Fig 2, left) The noradrenaline excretion rose markedly in the cold whereas only a minor increase in adrenaline excretion was observed The situation was different in the hypothyroid animals Their noradrenaline excretion in the cold rose only slightly above the level of the controls , but their adrenaline excretion reached a level more than 5 times higher than that of the control goats (Fig 2 right) To maintain thermal homeostasis in the cold the hypothyroid goats apparently had to compensate the lack of thyroid hormone by a conspicuous increase in adrenaline secretion Since denervation of the adrenal medulla in the euthyroid goat V abolished the moderate rise in blood glucose that occurs during hypothalamic cooling it can be concluded that this hyperglycemia is due to an increased adrenaline secretion and that the most likely explanation for the marked hyperglycemia that developed in the cold exposed hypothyroid goats is their extremely high adrenaline secretion

Also at room temperature the catecholamine excretion of the hypothyroid goats I III and IV was higher than in the controls In spite of the high adrenaline secretion the basic blood glucose level of these animals was within the normal range (40—55 mg/100 ml) It may be that the maintenance of a normal blood glucose level during lack of thyroid hormone requires an increased catecholamine secretion This is also indicated by earlier studies which have shown that hypothyroid rats respond to insulin induced hypoglycemia (Hokfelt 1951) or to cellular glucopenia induced by 2-deoxy glucose (Johnson 1965) with a higher adrenaline secretion than euthyroid rats

The importance of the sympathetic system in thermogenesis has also been demonstrated by the use of ganglionic blocking agents Hsieh Carlson and Gray (1957) have found that the administration of a ganglionic blocking agent eliminates the metabol c response to cold in rats Also the temperature rise occurring during local cool ng of the thermoregulatory "centre of the goat is prevented bv pre treatment with a ganglionic blocking agent and the shivering response of the animals becomes markedly reduced Under such circumstances an infusion of adrenaline or of noradrenaline causes the reappearance of full strength shivering and a rise in body temperature (Andersson *et al* 1964 b) Thus a high level of catecholamines in the blood seems to facilitate thermal shivering in the goat This may be the explanation for the very accentuated shivering response to cold observed in the hypothyroid animals

Since the absence of adrenaline secretion observed after splanchnicotomy d d not prevent shivering and the rise in body temperature during local cooling of the thermoregulatory centre it must be concluded that noradrenaline alone is sufficient to maintain cold stimulated thermogenesis and shivering in the euthyroid animal It remains to be sl own whether this holds true also in the markedly hypothyro d goat with its extremely high adrenal ne secretion in the cold

Th s work was supported by the Swed sh Med cal Research Counc l Project No 14 503 — 0

References

ANDERSSON, B, L EKMAN C. C GALE and J W SUNDSTEN, Control of thyreotrophic hormone (TSH) secretion by the "heat loss center" *Acta physiol scand* 1963 a *59* 12—33

ANDERSSON, B, C C GALE, B HOKFELT and A OHGA, Relation of the preoptic temperature to the function of the sympathico-adrenomedullary system and the adrenal cortex *Acta physiol scand* 1963 b *61* 182—191

ANDERSSON, B, C C GALE and B HOKFELT, Studies of the interaction between neural and hormonal mechanisms in the regulation of body temperature In "Major problems in neuro-endocrinology", ed by E BAJUSZ and G JASMIN S Karger, Basel/New York 1964 a pp 42—61

ANDERSSON, B, A H BROOK, C C GALE and B HOKFELT, The effect of a ganglionic blocking agent on the thermoregulatory response to preoptic cooling *Acta physiol scand* 1964 b *61* 393—399

BARKER, S B and M J HUMPHREY, Clinical determination of protein bound iodine in plasma *J clin Endocr* 1950 *10* 1136—1141

CARLSON, L D Non shivering thermogenesis and its endocrine control *Fed Proc* 1960 *19* Suppl 5 25—30

EKMAN, L, Thyroidectomy of the goat *Acta physiol scand* 1965 *65* 331—336

EULER, U S v and F LISHAJKO, Improved technique for the fluorimetric estimation of catecholamines *Acta physiol scand* 1961 *51* 348—356

FINDLAY, J D and D ROBERTSHAW, The role of the sympatho-adrenal system in the control of sweating in the ox (Bos taurus) *J Physiol* (Lond) 1965 *179* 285—297

HART S J, Physiological responses to cold in nonhibernating homeotherms In "Temperature its measurement and control in science and industry" Vol 3, Part 3 ed by C M HERZFIELD and J D HARDY Reinhold publ corp New York 1963 pp 373—406

HSIEH, A C. and L D CARLSON Role of adrenaline and noradrenaline in chemical regulation of heat production *Amer J Physiol* 1957 *190* 243—246

HSIEH, A C, L. D CARLSON and G GRAY, Role of sympathetic nervous system in the control of chemical regulation of heat production *Amer J Physiol* 1957 *190* 247—251

HOKFELT, B, Noradrenaline and adrenaline in mammalian tissues *Acta physiol scand* 1951 *25* Suppl 92

[illegible] hyroid and

RING, G C, The importance of the thyroid in maintaining an adequate heat production during exposure to cold *Amer J Physiol* 1942 *137* 582—588

SELLERS, E A and S S YOU, Role of the thyroid in metabolic responses to a cold environment *Amer J Physiol* 1950 *163* 81—91

Acta physiol. scand. 1967. 69. 119—120

From the Department of Histology, Karolinska Institutet, Stockholm Sweden

Electron Microscopic Studies on Brain Slices from Regions Rich in Catecholamine Nerve Terminals

By

TOMAS HOKFELT

Strong evidence has been obtained for the view that noradrenaline (NA) in adrenergic nerves is stored in high concentrations in subcellular particles Electron microscopic studies have shown that small granular vesicles (type 2 and 3 according to Grillo and Palay 1962) are present in such nerves and it has been suggested that these vesicles are identical with NA storage particles (cf Richardson 1964) So far it has not been possible to demonstrate such vesicles in the central nervous system (CNS), not even in regions which according to fluorescence microscopic studies contain a large number of monoamine nerve terminals (Fuxe, Hokfelt and Nilsson 1965) Evidence was obtained from these studies that at least the main part of brain monoamines is stored in structures which with routine fixation methods (glutaraldehyde and osmic acid) appear as small agranular vesicles (size about 500 Å) (Fuxe, Hokfelt and Nilsson 1965) In studies by other workers the large granular vesicles (type 1 according to Grillo and Palay 1962) have been considered to be the sites of NA storage in axons and synapses of the CNS

In a recent paper Richardson (1966) reported the successful use of potassium permanganate as fixative for the demonstration of small granular vesicles in the adrenergic nerve terminals of rat and mouse iris In these tissues it was previously difficult to demonstrate this type of vesicles In the present study this technique has been used for studies on the central nervous system

Thin brain slices from the caudate nucleus putamen and the hypothalamus (the periventricular region) were incubated for 20 min in a physiological buffer solution containing α methyl-NA in order to increase the catecholamine stores (Hamberger and Masuoka 1965) As a control, slices from reserpine pretreated rats (5 mg/kg, 10 hrs) were incubated in amine free buffer Immediately afterwards the slices were fixed in an ice-cold 3% potassium permanganate solution An RCA-EMU-2c electron microscope was used

Although deficiency in morphology was obvious a large number of vesicles containing a dense core was observed in certain boutons in both areas investigated

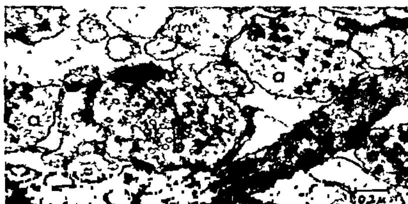


Fig 1 Slice of the caudate nucleus putamen of rat after incubation in α methyl NA containing buffer-solution Three boutons (a) contain small granular vesicles One bouton (b) contains only small agranular vesicles

(Fig 1) These dense core vesicles were about 500—600 Å in diameter, i e about the same size range as the small granular vesicles (type 2 and 3) as defined by Grillo and Palay (1962) In the majority of the boutons, however, no small granular vesicles could be found but only small agranular vesicles After incubation of slices from reserpine treated animals in an α-methyl NA free medium no small granular vesicles were observed Thus, the present data indicate that the small granular vesicles observed contain catecholamines In view of this it may be that also in the central nervous system small granular vesicles are storage sites of monoamines, although usual fixation methods fail to reveal this type of vesicles

The skilful technical assistance of mrs Waldtraut Hiort is gratefully acknowledged
This work has been supported by the Swedish State Medical Research Council (12x 714 02) For generous supply of reserpine we thank CIBA Ltd Stockholm

References

Fuxe K T Hokfelt and O Nilsson A fluorescence and electronmicroscopic study on certain brain regions rich in monoamine terminals *Amer J Anat* 1965 *117* 33—46
Grillo M and S L Palay Granule-containing vesicles in the autonomic nervous system *Electron microscopy* 1962 Ed S S Breese Jr Academic Press N Y 1962 2 U 1
Hamberger B and D Masuoka, Localization of catecholamine uptake in rat brain slices *Acta pharmacol* (Kbh.) 1965 *22* 363 368
Richardson K C The fine structure of the albino rabbit iris with special reference to the identification of adrenergic and cholinergic nerves and nerve endings in its intrinsic muscles *Amer J Anat* 1964 *114* 173—206
Richardson K C Electron microscopic identification of autonomic nerve endings *Nature* (Lond) 1966 *210* 756

Acta physiol. scand. 1967. 69. 121—122

From the Department of Clinical Neurophysiology, University of Uppsala Sweden

Mechanoreceptor Activity Recorded Percutaneously with Semi-Microelectrodes in Human Peripheral Nerves

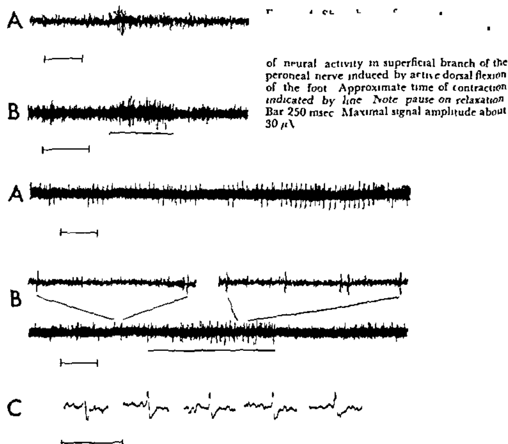
By

K.-E. Hagbarth and Å. B. Vallbo

Ordinary methods for percutaneous recording from human peripheral nerves require well synchronized nerve volleys yielding compound action potentials (Dawson and Scott 1949, Sears 1959, Gassel and Diamantopoulos 1965) There is little doubt, however, that any percutaneous method which permits recording also of asynchronous, dispersed activity in selected groups of afferent nerve fibres — like the group 1A afferents — would highly increase the possibilities to analyze peripheral as well as central neural functions in man The present results indicate that such recordings can be made with moderate technical facilities and with relatively minor discomfort for the subject

The electrodes were coated (Voltalac 435) tungsten wires with an uninsulated tip of 10—40 μ and a resistance of 0 01—1 Mohm They were inserted manually through the skin into the underlying nerve trunk and the position was adjusted until any kind of neural discharge appeared in response to mechanical stimuli applied distally within the receptive field of the nerve The signals were fed into a differential high-gain pre amplifier (Tektronix 122), displayed on an oscilloscope and also recorded on tape The stored signals were afterwards displayed (with reduced tape speed) on an inkwriter (Elema Mingograph) The nerves recorded from were the ulnar nerve (at the elbow), the peroneal and the tibial nerve (in the popliteal fossa) The authors were the subjects in all experiments so far

The type of afferent discharges most commonly seen were short bursts of high frequency impulses appearing in response to light taps within restricted distal fields or sudden passive movements of one or more digits (Fig 1 A) Besides such phasic discharges more prolonged neural activity was frequently recorded, particularly in response to active or passive distal joint movements Fig 1 B) Occasionally, recordings were obtained in which single unit discharges could be identified The "spontaneous" activity shown in Fig 2 A contains diphasic signals of fairly uniform shape and amplitude (Fig 2 C) repeated at a frequency of about 8/sec This frequency increased to about 25/sec when a moderate pressure was applied within a restricted area on the lateral side of the leg and a distinct pause appeared on



... of neural activity in superficial branch of the peroneal nerve induced by active dorsal flexion of the foot. Approximate time of contraction indicated by *line*. *Note pause on relaxation*. Bar 250 msec. Maximal signal amplitude about 30 μV.

Fig. 2. Activity recorded in superficial branch of the human peroneal nerve. *A*. Spontaneous activity. Bar 1.0 sec. *B*. Lower trace. Activity enhanced by moderate pressure applied locally within receptive field laterally on the leg. Stimulus indicated by line. Parts of the record (as indicated by diverging lines) are shown with extended time scale (1—20) in the upper traces. Bar 1.0 sec. *C*. Oscilloscope recordings of single discharges shown in A. Bar 10 msec. Signal amplitude about 30 μV.

cessation of the pressure (Fig. 2 B). The unit could not be activated by slight touch or by pressure applied to other areas of the leg. Several such selective recordings have been obtained and a systematic study of various kinds of superficial and deep mechanoreceptors has begun.

References

Dawson, G. D. and J. W. Scott: The recording of nerve action potentials through skin in man. *J. Neurol. Neurosurg. Psychiat.* 1949. *12*. 259—267.

Gassel, M. M. and E. Diamantopoulos: Nerve potential recording during electrically and mechanically evoked monosynaptic reflexes in man. *Nature* (Lond.) 1965. *208*. 1004—1005.

Sears, T. A., Action potentials evoked in digital nerves by stimulation of mechanoreceptors in the human finger. *J. Physiol.* (Lond.) 1959. *148*. 30 P-31 P.

Acta physiol. scand 1967. 69. 123—124

From the Department of Clinical Neurophysiology, University of Upsala, Sweden

Impulse Patterns in a Muscle Nerve during Voluntary Contraction in Man

By

Å B Vallbo

With the recording method previously described (Hagbarth and Vallbo 1966) it was often possible to observe how neural activity appeared in the peroneal nerve during voluntary contractions of the peroneal muscles The results suggest that much of this activity was derived from mechanoreceptors in the muscles It seems, therefore, that an analysis of the signals may help to elucidate some of the problems concerning muscle afferents and motor control in man

The neurograms shown in Fig 1 were all obtained from the same recording site in the superficial branch of the peroneal nerve (in the popliteal fossa, Afferent activity could be recorded when pressure was applied over the relaxed peroneal muscles or when these muscles were stretched by passive supination of the foot Neural activity of similar appearance could be induced also by slow active — but not by passive — pronation of the foot Simultaneous EMG recordings from the peroneal muscles during such slow voluntary contractions showed 1) that the neural activity often was intense even when only a few motor units were activated and 2) that the neural discharge started before and lasted 0 5—1 sec longer than the EMG activity (Fig 1 A) Sometimes the activity in the nerve showed a maximum at the beginning of the contraction and then gradually subsided until a second burst appeared This burst coincided with the passive drop of the foot back to the resting position (Fig 1 B) Fig 1 C shows two examples of the activity seen when a fast voluntary contraction of the peroneal muscles was performed The most striking feature of these records is a distinct pause in the resting activity of the nerve, appearing shortly after the EMG activity — at the approximate time of the twitch

It seems likely that the resting activity as well as the activity induced by passive muscle stretch was derived from muscle spindles which were unloaded by the fast voluntary contractions Motor impulses probably contributed to the enhancement of the neural activity observed during slow voluntary contractions but the major part of this discharge might well be derived from group I A afferents activated via the gamma loop

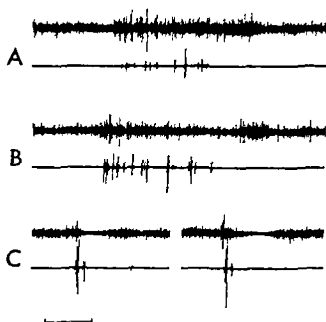


Fig 1 Activity in superficial branch of the peroneal nerve in man recorded at knee level with electrode inserted into the nerve (upper trace) and EMG activity recorded with bipolar needle electrodes in the peroneal muscles (lower trace)
A Slow voluntary contraction
B Slow voluntary contraction which stops abruptly giving a fast passive drop of the foot
C Fast voluntary contractions Bar 0 5 sec

Reference

HAGBARTH, K E and VALLBO Å B Mechanoreceptor activity recorded percutaneously with semi microelectrodes in human peripheral nerves Acta physiol scand 1967 *69* 121—122

Acta physiol scand. 1967. 69 125—126

From the Department of Histology Karolinska Institutet Stockholm Sweden

Ultrastructural Studies on Adrenergic Nerve Terminals in the Albino Rat Iris after Pharmacological and Experimental Treatment

By

TOMAS HOKFELT

Rat iris provides a good model tissue for pharmacological and experimental studies on the autonomic nervous system as shown e g by MALMFORS (1965) in fluorescence microscopic studies Previous studies at the ultrastructural level, however, have been hampered by the difficulties to distinguish between cholinergic and adrenergic nerve terminals, since the small granular vesicles (type 2 and 3 according to GRILLO and PALAY 1962), which are characteristic of sympathetic adrenergic nerve terminals could not be demonstrated This problem has partly been solved by the use of a special, glutaraldehyde fixation procedure (HOKFELT 1966) Until now, however, the potassium permanganate fixation as described by Richardson (1966) seems to give the most constant and best reproducible results and this technique has been used in the present study

Albino rats were subjected to the following pharmacological and experimental treatments (1) Reserpine (10 mg/kg i p 12 hrs (2) a methyl p-tyrosine methylester (H 44/68), a potent catecholamine (CA) synthesis inhibitor (500 mg/kg i p, 17 hrs) in combination with unilateral preganglionic electric stimulation (20/sec 80 min) of the sympathetic nerves supplying the iris (3) sympathectomy (b lateral [illegible] methyl noradr[illegible] of each grou[illegible] chloride (10 m[illegible] intraocular injection of ice-cold 3 % potassium permanganate solution An RCA EMU 2c electron microscope was used

As previously described (Hokfelt 1966) two types of nerve terminals are found in the dilator muscle of the rat iris One type is predominantly filled with small agranular vesicles while the other type mainly contains small granular vesicles of about 500 Å in diameter These two types probably represent the cholinergic and adrenergic nerve terminals respectively

After administration of reserpine, which is known to deplete monoamine stores in the nervous system almost all vesicles with a diameter of about 500 Å were agranular, which was also the case after preganglionic stimulation, provided that the resynthesis had been inhibited with in this case H 44/68 (Fig 1) On the not stimulated side, however many small granular vesicles remained (Fig 2) After sympathectomy (42 hrs) only agranular vesicles were seen These results agree with biochemical and fluorescence microscope studies

To determine whether exogenous NA could cause the reappearance of the small granular vesicles, a methyl NA which is not broken down by monoamine oxidase, was given It was found that in animals pretreated with reserpine or subjected to bilateral sympathectomy the administration of a methyl NA did not cause the reappearance of the small granular vesicles However in animals treated with H 44/68 in



Fig 1 Dilator muscle of iris of H 44/68 treated rat, stimulated side Almost no small granular vesicles can be seen in any terminal Scale 0 3 μ



Fig 2 Dilator muscle of iris of H 44/68-treated rat, non-stimulated side A rather large number of small granular vesicels can be seen in one terminal (a) Scale 0 3 μ

combination with stimulation many small granular vesicles could be observed in certain nerve terminals after receiving α-methyl-NA

The present findings leave little doubt that the small granular vesicles represent the NA storage granules and evidence has been obtained that NA is released from these granules upon prolonged nerve stimulation There exists strong evidence from previous work that at least two uptake mechanisms exist in the adrenergic neurons (*cf* Malmfors 1965), one is Mg^{++}-ATP dependent and present at the level of the amine granules and is reserpine sensitive, the other is probably localized at the level of the nerve cell membrane The present results give morphological evidence that the uptake mechanism of the amine storage granules is blocked by reserpine (see also Van Orden, Bensch and Giarman 1966) Thus, the amines known to exist in the terminals after α-methyl-NA (0 1—0 5 mg/kg) administration to reserpine pretreated animals (Malmfors 1965) probably lie extragranularly The results also show that the uptake-storage mechanism of the amine granules is intact after depletion induced by electrical stimulation plus synthesis inhibition, since in this case the small granular vesicles reappear after α methyl-NA administration

Hiort is gratefully
ie' and a research
nerous supplies of
H Corrodi, AB

References

GRILLO, M and S L PALAY, Granule containing vesicles in the autonomic nervous system *Electron Microscopy* Ed S S Breese Jr Academic Press, N Y 1962 2 U—1

HOKFELT, T, Electron microcopic observations on nerve terminals in the intrinsic muscles of the

microscopy

ings *Nature* (Lond) 1966 *210* 756

VAN ORDEN L. S, K G BENSCH and N J GIARMAN, Inhibitory effect of excess norepinephrine in the extragranular compartment *Fed Proc* 1966 *25* 260

Acta physiol scand. 1967. 69. 127—128

From the Departments of Pharmacology and Histology, Karolinska Institutet, Stockholm Sweden

Excessively Growing Monoamine-Containing Nerves in Mice during Treatment with a Nerve Growth Factor (NGF)[1]

By

WOLFGANG HAMMER, KARL-AXEL NORBERG, LARS OLSON and FOLKE SJOQVIST

Growth and differentiation of sympathetic neurons are influenced by a specific nerve growth factor (NGF) present in many tissues, especially the submaxillary gland of the adult male mouse (*see* Levi Montalcini 1964) The fluorescence microscopical technique of Falck and Hillarp (1962) has now been used to study the effect of NGF on adrenergic neurons in iris, salivary glands and paravertebral ganglia

Material and methods

[illegible]

iris (prepared according to Malmfors 1965), submaxillary and sublingual glands and paravertebral ganglia (superior cervical, stellate, selected thoraco-lumbar)

Results and discussion

The dilator muscle of the iris is supplied with a network of varicose adrenergic nerve terminals forming a ground plexus (Malmfors 1965) Each strand of the plexus usually contains one single fibre in the mouse (Fig 1, Malmfors 1965) In the NGF-treated mice (Fig 2) the strands of the plexus often contained up to 5 or more fibres and the general pattern of the ground plexus appeared less regular than normally The number of non terminal axons was also considerably increased (Olson 1966)

The serous acini of the submaxillary gland are supplied with an adrenergic ground plexus, while the mucous acini of the sublingual gland and the larger excretory ducts of both glands lack this innervation (*cf* Norberg and Olson 1965) In the submaxillary glands of the NGF treated mice a very pronounced increase in the size and number of the interlobular monoamine-containing axons and an increased density of the adrenergic innervation to the blood vessels were observed The ground plexus of the parenchyma had a more prominent appearance In the sublingual gland a similar increase of fluorescent nerves could be seen in the interlobulary regions and around the blood vessels Interestingly, adrenergic nerve terminals were also detected around the normally non innervated mucous acini

The paravertebral ganglia of control mice showed normally fluorescent cell bodies but very few varicose terminals under the conditions used (*cf* Norberg and Hamberger 1964) After NGF-treatment however, terminals consistently appeared surrounding the cell bodies in basket like synaptic structures

[1] A preliminary account of part of this investigation has appeared Olson 1966)

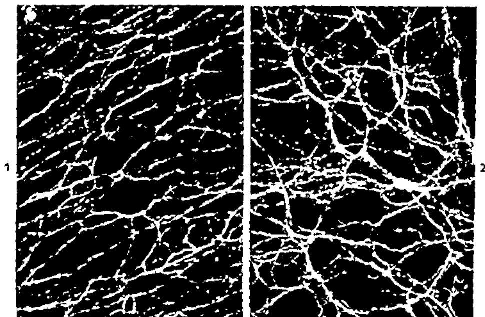


Fig 1 Iris dilator muscle untreated mouse The adrenergic nerve terminals run mainly single in the strands of the autonomic ground plexus ×220

Fig 2 Iris, dilator muscle NGF treated mouse The general pattern of the ground plexus is partly disorganized and the strands of the ground plexus consist often of several nerve terminals running together ×220

The results confirm and extend those of Levi Montalcini (1964), who first described the excessive growth of symphathetic nerves, which takes place during treatment with NGF Evidently, NGF markedly stimulates the outgrowth of adrenergic fibres both to tissues normally innervated by adrenergic nerves and to tissues normally lacking such innervation The increased density of adrenergic terminals in the sympathetic ganglia is of particular interest It has been shown previously that many autonomic ganglia contain adrenergic nerve terminals (cf Norberg and Sjoqvist 1965) The opportunity that now exists to increase their number with NGF may open new possibilities for studies of their functional role in ganglionic transmission

The authors are much indebted to prof R Levi Montalcini Dept Zool Washington Univ St Louis Mo and Dr D C Edwards Wellcome Res Lab Beckenham England for generous supplies of NGF

The investigation was supported by grants from the Swedish Medical Research Council (B66—257 and B67—12x—714—02) the National Institutes of Health Bethesda U S A (GM—13978—01) and Magn Bergvalls Stiftelse

References

... nd and its

... ol scand 1965 ...

NORBERG, K A and ... me characteristics revealed by histochemical studies on the intraneuronal distribution of the transmitter Acta physiol scand 1964 63 Suppl 238 1—42

NORBERG K A and L OLSON Adrenergic innervation of the salivary glands in the rat Z Zellforsch 1965 68 183—189

... on of ganglionic trans-

... e growth promoting ... 1966 68 Suppl 277

Acta physiol. scand. 1967. 69. 129—133

From the Metabolic Division, Department of Pharmacology and the Deparment of Zoophysiology, University of Gothenburg, Sweden

The Effect of H^+ — and Lactate Ions on the Electrical Activity and Content of High Energy Phosphate Compounds of Taenia Coli from the Guinea Pig

By

GUNNAR AABERG, ELLA MOHME-LUNDHOLM and NANDOR VAMOS

Received 25 May 1966

Abstract

AABERG, G., E. MOHME-LUNDHOLM and N. VAMOS. *The effect of H^+ — and lactate ions on the electrical activity and content of high energy phosphate compounds of taenia coli from the guinea pig.* — Acta physiol. scand. 1967. 69. 129—133.

[illegible]

on the electrical activity of the membrane

It has long been known that acids in a low concentration have a relaxing effect on smooth muscle. Increasing acidity in the tissues is assumed to be partly responsible for vascular dilatation on exercise and after ischaemia. The relaxing effect of catecholamines on smooth muscle has been ascribed to a stimulation of the carbohydrate metabolism and increased lactate production in the muscle (review Mohme-Lundholm 1953).

The mechanism for the relaxing effect of the H^+-ions is not known. *A priori* such an effect may be induced 1. by inhibition of the electrical activity of the muscle membrane, 2. by influence on the coupling of the excitation and contraction processes, 3. by inhibition of the metabolism so that the concentration of high energy phosphate compounds is insufficient for the contraction process to take place.

There are methods by which the modes of influence mentioned in points 1 and 3 can be demonstrated. At present the mechanism in point 2 can can only be shown as a theoretical probability by a process of elimination.

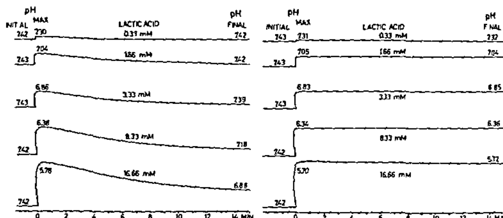


Fig 1 pH changes of Krebs bicarbonate buffer solution at 37° C on addition of lactic acid in different concentrations In the left part of the Fig the solution was aerated with 3 % CO_2 in O_2 during the whole experiment In the right part aeration was stopped 1 min before the addition of lactic acid and the Krebs solution was covered by a larger of liquid paraffin

In experiments on taenia coli from the guinea pig we have studied the effect of lactic, acetic and carbonic acids in relaxation inducing concentrations on the electrical activity of the smooth muscle membrane, and also the effect of lactic acid on the content of high energy phosphate compounds, in an attempt to localize the point of attack of the relaxing effect of these acids on smooth muscle

Method

A guinea pig weighing 200 300 g was killed by a blow on the neck Pieces of taenia coli about 20 mm long were carefully dissected out and immersed in Krebs bicarbonate solution

Electrical record ng In these experiments (about 30 animals) a piece of taenia coli was mounted in a sucrose gap apparatus as described by Burnstock and Straub (1958) and modified by Axelson (1961) The action potential was recorded on a Grass polygraph and the muscle tension with a Grass force transducer (FTO_3) One end of the muscle specimen was depolarized with K Krebs solution while the other was suspended in Krebs solution gassed with 3 % CO_2 in O_2 giving *a pH of 7 42 pH changes in the Krebs solution at 37 C were recorded with a Radiometer pH* ... rapidly ... eft the ... olution ... f lact c ...) In

Fig 1 is also shown the effect on pH of covering the solution with liquid paraff n

Metabolic experiments In these experiments pieces of taenia coli about 20 mm long were mounted by means of silk thread in a tissue bath containing 30 ml Krebs solution gassed with 5 CO_2 in O_2 The tension of the muscle was recorded with a Grass force transducer on a Grass polygraph The length of the specimens were adjusted so that they gave an initial tension of about 1 g After ... ation ... After ... cho- ... ect ... line stimulated speci iens ... 20—30 sec ... pronounced relaxat ... mens were frozen in Freon 11 at — 80 C The specimens which weighed 30—50 mg were then homogenized in 0 5 ml 6 % perchloric acid The extract was centrifuged the excess of perchloric acid in the solution was precipitated with potassium carbonate and the potassium perchlorate was then sedimented by centrifugation The ATP concentration in the extract was then determined enzymatically according to Adam (1962) creatine phosphate (crP) according to Lundholm and Vamos (1967) and lactic acid according to Lundholm Mohme Lundholm and Vamos (1963)

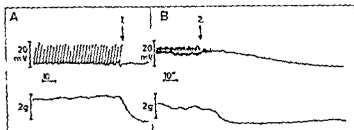


Fig 2 Taenia coli from the guinea pig The upper curve represents the electrical activity of the membrane, and the lower curve the tension
2A At arrow 1 the Krebs solution is changed to one containing 0 1 mmole/l lactic acid
2B At arrow 2 the Krebs solution is changed to one containing 8 mmoles/l lactic acid

Results

The effect of H^+ ions on the electrical activity of the membrane From Fig 2A which shows a typical experiment it can be seen that lactic acid already in a final concentration 0 1 mmole/l (maximal pH change 0 05 units) had an inhibitory effect on the electrical activity and reduced the tension In a higher concentration (8 mmoles/l, Fig 2B) lactic acid also induced hyperpolarization of the membrane After some min the electrical activity of the membrane however returned with a reduced frequency and in a irregular manner, the tension also increased irregularly Not until the specimen was resuspended in ordinary Krebs solution did the initial electrical activity and tension return There was no definite differences in the behavior of muscle when the experiments were done with a Krebs solution covered with liquid paraffin In general similar results were obtained on the addition of acetic acid or by increasing the content of CO_2 in the gassing mixture from 3—12 % (pH change 7 42 → 7 14)
The effect of lactate and acetate on the electrical activity and tension of the muscle When sodium lactate was added in a concentration of 1 mmole/l the frequency and amplitude of the action potentials increased (Fig 3A) and the tension also increased at the

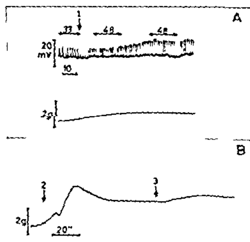


Fig 3A Taenia coli from the guinea pig
A At arrow 1 1 mmole l sodium lactate of pH 7 3 is added The action potentials increase in frequency from 33 min to a maximum of 48 min and at the same time the amplitude increases and the tension rises
3B At arrow 2 145 meq l K Krebs is added and at arrow 3 3 1 mmoles l lactic acid

TABLE 1 The effect of 10^{-3} M lactic acid in Krebs solution on the lactic acid content and high energy compounds ATP and CrP in taenia coli Mean ± S E M of 13 tests Concentration in μ moles g wet weight

	ATP	CrP	Lactic acid
Basal values	1 1 ± 0 20	1 2 ± 0 23	7 6 ± 0 1
Changes from basal values after lactic acid	— 0 03 ± 0 07	— 0 19 ± 0 16	— 0 17 ± 1 1

same time No effects of sodium lactate were observed, however, on carbacholine-stimulated muscle Sodium acetate in a concentration of 3 mmoles/l had some relaxing effect NaCl in equivalent concentrations had no effect, and therefore the Na^+-ion does not appear to have played any role in these experiments

The effect of pH changes and adrenaline on the muscle tension in K^+-rich solution In a Krebs-Henseleit bicarbonate solution in which all Na^+-ions had been replaced by K^+-ions, the muscle contracted According to Evans, Schild and Thesleff (1958), the smooth muscle membrane is depolarized in such K^+-rich solution A reduction of the pH by adding lactic acid or by increasing the CO_2 tension of the solution resulted in no relaxation of the K^+ depolarized muscle, on the contrary an increase of the tension was observed (Fig 3B) The relaxant effect of adrenaline was almost completely eliminated in K -rich solution Sodium lactate had no effect on the tension of K^+-depolarized muscle

The effect of lactic acid on the contents of high energy phosphate compounds and lactic acid in taenia coli When lactic acid in a concentration of 1 mmole/l was added to muscle previously treated with carbacholine, almost total relaxation of the muscle was observed The concentrations of ATP and creatine phosphate remained unchanged No increase was seen in the lactate concentration in the muscle (Table 1) This latter finding should be considered in the light of the fact that the quantity of lactic acid added only increased the lactate content in the suspension solution to 1 mmole/l, while the basal content of the muscle was, on the average, 7 6 mmoles/kg

Discussion

The relaxant effect of H -ions on taenia coli was combined with an inhibitory effect on action potentials and possibly hyperpolarization of the membrane When action potentials returned, the tension increased No definite effect of lactic acid on the content of high energy phosphate compounds was shown In K^+-depolarized muscle a reduction of the pH had a tension increasing effect These results indicated that the relaxant effect of the H ions on the smooth muscle cell was associated with its effect on the electrical activity of the muscle membrane The possibility that the H^+ ion influenced, in addition, the reactions connecting the excitation and contraction processes cannot be excluded, however The lactate ion had a stimulatory effect on the action potentials and induced an increase in the tension The acetate ion, on the other hand, had a relaxant effect It is thus conceivable that the two components

in lactic acid counteracted each other's effects, while the H⁺-ion and the acetate ion both had a relaxant effect. It was found by Bulbring (1960) in experiment on taenia coli that adrenaline inhibited the action potentials and hyperpolarized the muscle membrane and relaxed the muscle. In K^+-rich solution when the membrane probably was depolarized (Evans *et al.* 1958) the relaxing effect of adrenaline was almost completely eliminated. Since adrenaline stimulates the carbohydrate metabolism and increases the lactate content in smooth muscle, it does not seem improbable that the effect of adrenaline on the electrical activity of the muscle membrane could be, at least in part, a result of increased lactate production. Lundholm, Mohme-Lundholm and Vamos (1967) have also found that the phosphorylase-activating effect of adrenaline is blocked in K^+ depolarized skeletal muscle. Under certain conditions, however, the catecholamines may also relax depolarized muscle (Schild 1960), and it therefore seems probable that mechanisms other than blockade of the electric activity of the muscle membrane may also be of importance for the relaxant effect of the catecholamines. For further discussion on these problems, see Lundholm, Mohme-Lundholm and Svedmyr (1966).

This investigation was supported by grants from the Swedish State Medical Researach Council (U 285) and the Swedish National Association against Heart and Chest Diseases and the Science Faculty of the University of Gothenburg.

References

ADAM, H. Adenosin-5-triphosphate. Bestimmung mit Phosphoglycerat Kinase. In: *Methoden der enzymatischen Analyse* (H. U. Bergmeyer ed.) 1962. 539.

AXELSSON, J. Dissociation of electrical and mechanical activity in smooth muscle. *J. Physiol. (Lond.)* 1961. *158*. 381—398.

BROWN, G. L., M. GOFFART and M. V. DIAS, The effect of adrenaline and of sympathetic stimulation on the demarcation potential of mammalian skeletal muscle. *J. Physiol. (Lond.)* 1950. *111*. 184—194.

BULBRING, E. Biophysical changes produced by adrenaline and noradrenaline. In: *Adrenergic Mechanisms. Ciba Found. Symp.* (ed. by J. R. Vane, G. E. W. Wolstenholme and C. M. O'Connor) 276—286. Churchill Ltd. London 1960.

BURNSTOCK, G. and R. W. STRAUB. A method for studying the effects of ions and drugs on the resting and action potentials in smooth muscle with external electrodes. *J. Physiol. (Lond.)* 1958. *140*. 156—167.

EVANS, D. H. L., H. O. SCHILD and S. THESLEFF. Effects of drugs on depolarized plain muscle. *J. Physiol. (Lond.)* 1958. *143*. 474—485.

LUNDHOLM, L. and E. MOHME-LUNDHOLM. The carbohydrate metabolism and tone of smooth muscle. *Acta pharmacol.* (Kbh.) 1960. *16*. 374—388.

LUNDHOLM, L., E. MOHME-LUNDHOLM and N. SVEDMYR. The relation between some metabolic and pharmacological effects of the catecholamines. *Pharmacol. Rev.* 1966. 18. 255—272.

LUNDHOLM, L., E. MOHME-LUNDHOLM and N. VAMOS. Lactic acid assay with L(+)lactic acid dehydrogenase from rabbit muscle. *Acta physiol. scand.* 1963. *58*. 243—249.

LUNDHOLM, L., E. MOHME-LUNDHOLM and N. VAMOS. Blockade of the phosphorylase activating effects of adrenaline and cyclic 3',5'-AMP in K-depolarized rat diaphragm. Manuscript.

LUNDHOLM, L. and N. VAMOS. Enzymatic determination of creatine phosphate.

MOHME-LUNDHOLM, E. The mechanism of the relaxing effect of adrenaline on smooth muscle. *Acta physiol. scand.* 1953. 29. Suppl. 108. 1—63.

SCHILD, H. O. Effect of adrenaline on depolarized smooth muscle. In: *Adrenergic Mechanisms. Ciba Found. Symp.* ed. J. R. VANE, G. E. W. WOLSTENHOLME and C. M. O'CONNOR. 288—292. Churchill Ltd. London 1960.

Acta physiol. scand. 1967. 69. 134—139

From the Department of Physiology, University of Lund, Sweden

Choline Acetylase in Normal and Denervated Submaxillary Glands of Rats after Repeated Teeth Amputations

By

PER OHLIN and CARLOS JOSE PEREC[1]

Received 13 August 1966

Abstract

OHLIN, P and C J PEREC *Choline acetylase in normal and denervated submaxillary glands of rats after repeated teeth amputations* Acta physiol scand 1967 *69* 134—139

The size of the rat s submaxillary gland and the total activity of choline acetylase in the gland were markedly and similarly increased after repeated teeth amputations Preganglionic parasympathetic denervation caused an atrophy of the gland and a pronounced decrease in enzyme activity

The submaxillary gland of rats is supplied with both parasympathetic and sympathetic nerve fibres as shown by histological (Gljmstedt and Hillarp 1942, Norberg and Olson 1965) and physiological studies (Ohlin 1965) There is evidence that the two sets of nerves interfere with each other in different ways Thus, in cats the secretory response to parasympathetic stimulation or parasympathomimetics is modified by the sympathetic transmitter and contrariwise (Assarson and Emmelin 1964, Emmelin 1965) In cats and rats postganglionic sympathetic denervation of the submaxillary gland causes an increase in the choline acetylase activity of the postganglionic parasympathetic neurons (Nordenfelt 1964 a)

A marked and rapid increase in the size of the rat's submaxillary gland follows repeated amputations of the incisors The gain in gland weight is due to a hypertrophy of the acinar cells Wells *et al* 1959, Perec *et al* 1965) The glandular enlargement seems to be dependent both on the parasympathetic and sympathetic nerve fibres since it is less marked in parasympathetically or sympathetically denervated glands (Wells and Peronace 1964)

In the present investigation the effect of repeated teeth amputations on the parasympathetic neurons of the rat's submaxillary gland was studied by estimating the

[1] Fellow of the Consejo Nacional de Investigaciones Cientificas y Tecnicas Buenos Aires Argentina

activity of choline acetylase It was also studied to what extent the effect on the parasympathetic neurones of the gland was modified by sympathetic or parasympathetic denervation or both In addition the content of noradrenaline was determined in enlarged and control glands

A preliminary report of this paper was given at the XII Scandinavian Congress of Physiology, Turku (Ohlin and Perec 1966 a)

Methods

Sixty three male rats of a strain bred at this Institute were used They were given water and a pelleted diet (Anticimex 210) *ad libitum* the rats with amputated teeth received crushed pellets in milk The animals were weighed at the beginning and at the end of all experiments The body weight was 200—300 g when the experiments were started

Fifty four rats were used for the estimation of the choline acetylase activity, they were divided into eight groups

Group 1 3 rats Teeth amputations
Group 2 8 rats Chorda lingual section
Group 3 8 rats Teeth amputations + chorda lingual section
Group 4 3 rats Superior cervical ganglionectomy
Group 5 4 rats Teeth amputations + superior cervical ganglionectomy
Group 6 9 rats Chorda lingual section + superior cervical ganglionectomy
Group 7 9 rats Teeth amputations + chorda lingual section + superior cervical ganglionectomy
Group 8 10 rats Unoperated controls

Teeth amputations were made with a small bone cutting forceps for 14 days every two days all 4 incisors were cut in order to obtain a marked gain in weight of the submaxillary gland (Perec *et al* 1965) A preganglionic parasympathetic denervation was achieved by section of the ch [illegible] of the superior ce [illegible] The denerva tic [illegible]

[illegible] acetylase activity the submaxillary glands were carefully dissected cleaned and weighed and [illegible] the method described by [illegible] maxillary gland in some [illegible] for the enzyme estimation [illegible]

Results

Gland Weight

Teeth amputations were found to increase the size of the submaxillary gland significantly P 0 001 from 218 ± 6 8 (20)[1] mg to 318 ± 6 6 6) mg (Table I) The increase in gland weight was even more marked when expressed per unit body weight since teeth amputations were found to reduce the gain in body weight seen in the controls very likely due to a diminished food intake

Parasympathetic denervation alone caused a significant decrease P 0 001 in the weight of the gland The glandular size was slightly reduced by sympathetic

[1] Mean standard error of mean number of observations

TABLE I The activity of choline acetylase and the weight of the submaxillary gland in controls and in rats after teeth amputations, chorda lingual section and superior cervical ganglionectomy Values are mean ± S E M

	Number of glands	Gland Weight (mg)	Choline Acetylase µg ACh/hr/gland	Activity µg ACh/hr/g
Unoperated controls	20	218 ± 6 8	12 ± 1 2	320 ± 32
Teeth amputations	6	318 ± 6 6	23 ± 2 1	370 ± 27
Chorda lingual section	16	156 ± 6 0	8 5 ± 0 70	240 ± 40
Teeth amputations + chorda lingual section	16	280 ± 7 0	17 ± 1 6	310 ± 32
Superior cervical ganglionectomy	6	208 ± 11	16 ± 3 5	440 ± 57
Teeth amputations + superior cervical ganglionectomy	8	249 ± 9 9	23 ± 2 7	510 ± 35

TABLE II The activity of choline acetylase and the weight of the rat s submaxillary gland after chorda lingual section and superior cervical ganglionectomy on one side and after teeth amputations Values are mean ± S E M

	Number of glands	Gland Weight (mg)	Choline Acetylase µg ACh/hr/gland	Activity µg ACh/hr/g
Chorda lingual section + superior cervical ganglionectomy	9	150 ± 11	15 ± 0 2	430 ± 20
Normally innervated (contralateral)	9	200 ± 13	20 ± 1 7	410 ± 5
Teeth amputations + chorda lingual section + superior cervical ganglionectomy	9	130 ± 8 9	15 ± 0 8	490 ± 5
Normally innervated (contralateral) + teeth amputations	9	317 ± 35	26 ± 1 7	340 ± 10

denervation alone Teeth amputations increased the weight of the parasympathetically denervated glands markedly ($P < 0\ 001$) The weight of the sympathetically denervated glands was less markedly, though significantly ($P < 0\ 01$), increased after teeth amputations (Table I) The size of both parasympathetically and sympathetically denervated glands was significantly decreased ($P < 0\ 01$) It was not increased by repeated teeth amputations (Table II)

Choline Acetylase Activity

In control glands the total activity of choline acetylase was found to be 12 ± 1 2 (20) µg ACh/hr/gland, when expressed as concentration the activity was 320 ± 32 µg ACh/hr/g The total activity of choline acetylase was markedly increased ($P < 0\ 001$) after teeth amputations while the enzyme concentration was not significantly changed due to the pronounced gain in gland weight (Table I)

Parasympathetic denervation The total activity of chol
($P < 0.05$) in denervated glands The enzyme conc
though the decrease was not significant The total enzy
ically denervated glands was markedly increased (P
teeth amputations (Table I)

Sympathetic denervation After postganglionic sympathetic
of choline acetylase seemed to be increased, both wh
and as concentration (Table I) These results agree w
by Nordenfelt (1964 a) The total enzyme activity in
glands was increased by about 40 per cent after repeated

Parasympathetic and sympathetic denervation The total ac
denervated glands was 15 ± 0.2 (9) μg ACh/hr gla
0.8 (9) μg ACh/hr/ gland, following repeated teeth a

The denervations were unilateral The total enzym
glands was found to be decreased ($P < 0.05$) when
contralateral normally innervated glands, from 20 ±
ACh/hr/gland It may be noted that the total enzyme a
concentration, of the contralateral glands was higher (P
glands

Noradrenaline Content

The content was found to be 0.21 ± 0.02 (12) μg no
phied glands after repeated teeth amputations and 0.27
in the controls Thus the amount of noradrenaline
repeated teeth amputations Since teeth amputations in
in the weight of the glands (53.0 ± 1.4 per cent), the co
was markedly reduced The noradrenaline concentrat
controls and 0.60 ± 0.02 (12) μg noradrenaline g in t
difference is highly significant ($P < 0.001$)

Discussion

It is well-known that acetylcholine is the transmitter a
sympathetic nerves in salivary glands (Chang and G
Emmelin and Muren 1950 The acetylcholine synthesi
is localized in the cholinergic neurones of the glands (Nor
ity of choline acetylase was markedly increased after
in the rat's submaxillary gland The increase in enzyme
gain in gland weight It seems reas able to assume
activity reflects an extension of the pa asympathetic ne
other hand it may be noted that t tal amount of
was unchanged in hypertrophied g after repeated

Parasympathetic denervation causes an atrophy of salivary glands (Bernard 1864) The decrease in the total amount of choline acetylase in the rat's submaxillary gland after preganglionic parasympathetic denervation was even more pronounced than the reduction in the size of the gland, therefore the concentration of choline acetylase seemed lowered, as well After similar experiments on cats it has been suggested that the reduced enzyme activity is due at least in part to changes in the postganglionic parasympathetic neurones very likely caused by the loss of impulses from the central nervous system (Nordenfelt 1964 b) The remaining activity of choline acetylase in the decentralized parasympathetic neurones of the rat's submaxillary gland was found to be increased after repeated teeth amputations This finding indicates that the enzyme activity is not only affected by impulses in the preganglionic neurones

Parasympathetic decentralization causes an atrophy of the rat's submaxillary gland by about 25 per cent in one week (Ohlin and Perec 1966 b) When repeated teeth amputations were started one week after parasympathetic decentralization of the gland it caused a gain in gland weight similar to that observed in normally innervated glands Thus, preganglionic parasympathetic denervation of the rat's submaxillary gland does not inhibit the effect of repeated teeth amputations on the gland weight, as it has been previously reported (Houssay *et al* 1962, Wells and Peronace 1964) Similar results to those obtained after surgical denervation have been found following pharmacological denervation by treatment with atropine (Ohlin and Perec 1966 c)

The activity of choline acetylase is known to be increased in sympathetically denervated glands (Nordenfelt 1964 a) which was confirmed in the present investigation After postganglionic sympathetic denervation the total activity of choline acetylase was further increased in the glands following repeated teeth amputations The increase in enzyme activity roughly corresponded to the gain in gland weight It may be noted that the glandular enlargement of sympathetically denervated glands was much less pronounced than that of normally innervated and parasympathetically denervated glands It has been suggested (Nordenfelt 1964 a) that the increased activity of choline acetylase after sympathetic denervation is due to a further growth or collateral sprouting of the parasympathetic glandular neurones Then, it seems reasonable to assume on the basis of the present findings that the effect of repeated teeth amputations on the activity of choline acetylase in sympathetically denervated glands is due to a further extension of the postganglionic parasympathetic neurones in these glands

The weight of the sympathetically and parasympathetically denervated submaxillary gland of rats was not affected by repeated teeth amputations, nor was the activity of choline acetylase of these glands changed Thus, both the parasympathetic and sympathetic glandular nerves seem to be of importance for the gain in gland weight induced by repeated teeth amputations

We are indebted to Dr E Rosengren Institute of Pharmacology Lund for the estimations of noradrenaline

References

ASSARSON N and N EMMELIN, Leakage of transmitters in salivary glands *Brit J Pharmacol* 1964 *22* 119—125
BERNARD C, Du role des actions reflexes paralysantes dans le phenomène des secretions *J Anat (Paris)* 1864 *1* 507—513
BERTLER, Å, A CARLSSON and E ROSENGREN, A method for the fluorometric determination of adrenaline and noradrenaline in tissues *Acta physiol scand* 1958 *44* 273—292
BEZNAK, A B L, On the mechanism of the autacoid function of parasympathetic nerves *J Physiol* (Lond) 1934 *82* 129—153
CHANG, H C and J H GADDUM Choline esters in tissue extracts *J physiol* (Lond) *1933* *79* 255—285
EMMELIN N, Action of transmitters on the responsiveness of effector cells *Experientia* (Basel) 1965 *21* 57—65
EMMELIN, N and A MUREN, Acetylcholine release at parasympathetic synapses *Acta physiol scand* 1950 *20* 13—32
GLIMSTEDT G and N Å Hillarp, Über die Innervationsgebiete des Sympathikus und des Parasympathikus bei der Glandula submandibularis *Kgl Fysiogr Sällsk Handl* 1942 *53* 1—38
HOLSSAY A B, A A V PERONACE C J PEREC and O RUBINSTEIN, Role of the chorda tympani in submaxillary hypertrophy by incisor amputation in the rat *Acta physiol lat amer* 1962 *12* 153—165
NORBERG, K A and L OLSON Adrenergic innervation of the salivary glands in the rat *Z Zellforsch* 1965 *68* 183—189
NORDENFELT I, Choline acetylase in normal and denervated salivary glands *Quart J exp physiol* 1963 *48* 67—79
NORDENFELT I Choline acetylase in salivary glands of the rabbit, dog and rat after sympathetic denervation *Acta Univ Lund* II 1964 *10* 1—7
NORDENFELT, I Effect of preganglionic parasympathetic denervation on choline acetylase in postganglionic neurones *Acta Univ Lund* II 1964 *16* 1—7
OHLIN P Secretory responses of innervated and denervated submaxillary glands of rats *Acta Univ Lund* II 1965 *23* 1—12
OHLIN P and C PEREC Effects of repeated teeth amputations on the rat's submaxillary gland *Acta physiol scand* 1966 *68* Suppl 277
OHLIN P and C PEREC Effects of atropine treatment on the submaxillary gland of rats *Quart J exp physiol* 1966 *51* 196—201
OHLIN P and C PEREC To be publ
PEREC C J A B HOUSSAY, A A V PERONACE and F J DE HARFIN Submaxillary and retrolingual gland hypertrophy by different incisor amputations in the rat *J dent Res* 1965 *44* 683—683
WELLS H S J ZACKIN P GOLDHABER and P L MUNSON Increase in weight of the submandibular salivary glands of rats following periodic amputation of the erupted portion of the incisor teeth *Amer J Physiol* 1959 *196* 827—830
WELLS H and A A V PERONACE Synergistic autonomic nervous regulation of accelerated salivary gland growth in rats *Amer J Physiol* 1964 *207* 313—318

Acta physiol. scand. 1967. 69. 140—146

From Clinic II, Lillhagen Hospital, Lillhagen, and the Department of Pharmacology, University of Göteborg, Sweden

Presence of Free and Conjugated Noradrenaline in Human Cerebrospinal Fluid

By

S. J. Dencker, J. Haggendal and M. Ilves Häggendal

Received 13 September 1966

Abstract

Dencker, S. J., J. Haggendal and M. Ilves Haggendal. *Presence of free and conjugated noradrenaline in human cerebrospinal fluid.* Acta physiol. scand. 1967. *69.* 140—146.

The noradrenaline (NA) in the lumbar cerebrospinal fluid (CSF) was estimated by the trihydroxy indole reaction in patients with lumbar root syndromes or with mental diseases. The mean values

barrier is discussed.

The catecholamine levels in the cerebrospinal fluid (CSF) are of interest in the discussion of the effect of administered catecholamines on the central nervous system (CNS), as well as of the question whether the catecholamines in the CSF reflect the catecholamine metabolism in the CNS. See, for example, Euler (1956), Rothballer (1959) and Schain (1960).

The catecholamines in the CSF appear to have been studied to only a minor extent with chemical methods. But some reports have been published e. g. by Weil Malherbe and Liddell (1954) and Manger, Wakin and Bollman (1959).

The method generally used in earlier studies, the ethylenediamine condensation reaction has, however, been criticized as being unspecific and giving artificially high values (Euler 1956, Valk and Price 1956 and others). The catecholamine values in the blood plasma obtained with this method were thus generally higher than those obtained with methods based on the trihydroxyindole (THI) reaction. The CSF values of noradrenaline (NA) and adrenaline (A) reported in these earlier studies where somewhat lower than those found for the blood plasma. In the present study

the catecholamine levels in human CSF has been determined using the THI reaction

Parts of the study were presented at the XII Scandinavian Congress of Physiology, Turku 1966 (Haggendal 1966)

Material and methods

Lumbar CSF was obtained from 7 patients with lumbar root syndromes and from a mixed series of 72 inpatients of a mental hospital The samples were collected in ice-cold tubes containing 1 ml of 1 per cent ethylene diamine tetra acetate (EDTA) in 0 9 per cent sodium chloride or 1 ml of 1 N hydrochloric acid or 0 4 N perchloric acid The samples were deproteinized with perchloric acid and then treated as follows

a) Free catecholamines in a CSF sample generally 10 ml were adjusted to pH of about 5 5 and then absorbed by passage of the sample through a column of strong cation exchange resin (Dowex 50 W X8) After the column had been rinsed with buffer and water the catecholamines were eluated with hydrochloric acid oxidized and rearranged to fluorescent trihydroxyindoles and then estimated in an Aminco-Bowman spectrophotofluorometer D mercaptopropanol (BAL) and sodium sulfite were used to stabilize the fluorescence intensity

b) For estimation of the total (free and conjugated catecholamines the sample of about 10 ml was first hydrolyzed in N perchloric acid at 100 ° C for 10 min and then treated as described above

c) In some experiments non hydrolyzed CSF was passed through a column (on which the free catecholamines were absorbed) and collected together with a few ml of rinsing water and then hydrolyzed and after adjustment of the pH passed through a second column On this column the catecholamines liberated on hydrolysis were now absorbed They were estimated after elution as described above For details of the determination procedure see Haggendal (1963a)

The same procedure was used for estimating the free and conjugated NA and A in human blood plasma (e g Haggendal 1963 b and Carlsson et al 1966)

After some modifications (mainly reduction of all volumes in the THI reaction the dopamine (DA) was estimated by Carlsson and Lindqvist s (1962) modification of the method of Carlsson and Waldeck (1958

The catecholamines in brain were estimated in principally the same way in a tissue extract prepared with 0 4 N perchloric acid

Results

1 The free NA in the CSF in 68 of the 72 patients with mental diseases was 0 5 ± 0 03 μg l (mean ± S E M) In 4 cases the NA levels were remarkably high, i e 16 4 2 8 2 5 and 2 3 μg/l In the last case, however the major part of the catecholamines was adrenaline (A) Two of these patients were suffering from manic-depressive psychosis the other two were schizophrenics receiving treatment with chlorpromazine In the 7 patients with lumbar root syndromes the level was somewhat lower 0 2 ± 0 08 μg l

2 Except for the single case mentioned above, no A was found in the CSF

3 Except for some uncertain traces sometimes observed no DA could be detected in the CSF or the plasma The method for detecting DA was however not sensitive enough to exclude the occurrence of minute amounts of the substance

4 Twenty two of the CSF samples were subjected to acid hydrolysis (15 from patients with mental diseases and 7 from the group with lumbar root syndromes) The NA was increased in 19 cases and as a rule more distinct NA spectra were then obtained In one case the free NA level was high i e 2 8 μg l before and 2 9 μg l after acid hydrolysis was found In the other 21 cases the corresponding figures were 0 4 ± 0 04 μg l and 0 6 ± 0 07 μg l respectively

TABLE I Free and conjugated noradrenaline (NA) in cerebrospinal fluid (CSF) of males after oral

Case number	Age in years	Diagnosis	Treatment	Orally given NA
1	51	Psychopathy Alcohol addiction		2 mg × 3 for 3 days
2	26	Mental inferiority Hypomania	Chlorpromazine 600 mg × 3 Effontil®[a] 5 mg × 3	2 mg × 3 for 3 days
3	58	Chronic brain syndrome Alcohol addiction	Amphetamine 10 mg × 2	2 mg × 3 for 3 days
4	51	Mild depression		2 mg × 5 for 2 days
5	57	Mild depression		2 mg × 5 for 2 days
6	45	Muscle myalgia		2 mg × 5 for 2 days

[a] i e n ethyl norphenyl ephrinehydrochloride Benztropine 2 mg in the evening, was also given in

NA was also found when non-hydrolyzed CSF was passed through a column and then hydrolyzed and passed through a second column (see Methods) In this way the catecholamines liberated on hydrolysis were shown more directly

No conjugated A was found in the CSF The only case containing free A was unfortunately not hydrolyzed No significant amounts of conjugated DA could be demonstrated Two human brains were studied for conjugated catecholamines Tissue from the frontal cortex and the middle portion of the pons were examined 15 5 and 17 hrs after death in heart diseases in two men, aged 76 and 84 No certain increase of the NA or DA values were found after acid hydrolysis Thus no conjugated NA or DA was demonstrated in brain tissue The presence of minute amounts could not, however, be excluded

In order to ascertain whether the plasma levels of conjugated NA could influence the levels of conjugated NA in the CSF, 6 patients were given NA (Nor-Exadrin conc[8]) repeatedly by mouth (cf Haggendal 1963b)

Table I summarizes the diagnoses, the medical treatment, the doses and duration of NA given, and the levels of NA in the CSF after acid hydrolysis In 3 cases also the

load ng with NA

Interval in minutes between last NA dose and collection of sample	NA in plasma after acid hydrolysis (free and conjugated) in µg/l	NA in CSF (free) in µg/l	NA in CSF after acid hydrolysis (free and conjugated) in µg/l
60	5 8	0 2	0 9
60	5 7	0 5	0 6
60	22	0 2	1 2
50		0 8	2 8
40		2 1	3 5
40		0 2	2 0

th s case

plasma NA levels were estimated after acid hydrolysis The first 3 patients did not receive NA as often as the other 3 patients but showed relatively high levels of conjugated NA The conjugated NA levels in the CSF were *higher* after the larger doses

Discussion

The range of the individual values of NA in CSF in the patients with mental diseases was wide This may be due to some association between a certain mental disease and the catecholamine level in the CSF as well as to the influence of different types of treatment such as electroshock or drugs on the catecholamine level

In some cases the NA levels were too low to allow demonstration of the typical NA spectrum by the method used

The concentration of NA in normal human blood plasma at rest was found to be 0 3 + 0 11 µg l 13 cases) Normal plasma was not found to contain any A (Haggendal 1963) In a group (7 cases) of schizophrenics not treated with drugs (Carlsson *et al* 1967) the NA plasma concentration at rest was found to be 0 6 ± 0 07 µg l (9 cases) compared with 1 2 ± 0 16 µg l in 7 cases receiving chlorpromazine

The level was thus roughly the same in the CSF as in the blood plasma at rest, though it tended to be higher in the patients with mental diseases

Conjugation is a step in the catecholamine metabolism believed to take place in the liver or in the intestines, the bulk of a large dose of catecholamine given by mouth being excreted in the urine as conjugates (Richter 1940, Richter and MacIntosh 1941) This metabolic pattern thus differs from that after parenteral administration where 3 0 methylation plays an important role (Axelrod 1957, 1959) The circulating NA liberated from the sympathetic nerve terminals during muscular work, for example, appears to be metabolized chiefly according to this latter pattern After oral administration of A and NA large amounts of conjugated A and NA were found in the plasma (Haggendal 1963b, and unpublished results) In plasma collected during rest the ratio between the conjugated and free NA was 2—3 1 During physical activity the conjugated NA fraction did not rise as much as the free fraction

The origin of NA, free and conjugated, in the CSF is obscure Some authors contend that the blood/CSF barrier is impermeable to NA (see *e g* Euler 1956 for review), others that it is not (Schein 1960)

NA has been detected in solution surrounding electrically stimulated isolated spinal cords (Anden *et al* 1965) But the animals had been pretreated with high doses of a monoamine oxidase (MAO) inhibitor to protect the liberated NA from being destroyed within the spinal cord It is thus uncertain whether spinal cords from animals not treated with MAO inhibitors could liberate NA to the surrounding liquid

Some of the findings in this study suggest that NA may pass through the blood/CSF barrier

The levels of free and conjugated NA were generally somewhat lower in the CSF than in the blood plasma

Since no conjugated NA could be demonstrated in brain, the CSF component was probably not derived from the brain tissue But is must be observed that, for technical reasons, the presence of such minute amounts of conjugated NA in the CNS as in normal blood plasma could not be excluded

A was the dominating catecholamine in one case It seems unlikely that this A is derived from brain tissue since only very small amounts of A occur in the human brain tissue, while large amounts are occasionally found in the plasma

When the conjugated NA in the plasma was increased by oral loading with NA, the CSF NA after acid hydrolysis also increased It is possible that some barrier mechanism was overloaded by the high levels of conjugated NA in the plasma, but since conjugated NA is normally found in the CSF this suggestion cannot by itself explain the finding An admixture of blood components in the tapped CSF sample does not appear likely since 5 of the 6 samples contained no red cells and the sixth only 15 per 3 2 μl

The patients had different mental diseases That the increase in the conjugated NA values in CSF after oral loading should be due solely to the mental disease seems unlikely, since conjugated NA was also found in one patient without mental disease (the last case, Table I) and in the patients with a lumbar root syndrome How-

ever, in all these patients the blood/CSF barrier may for different reasons be impaired It would therefore be of interest to study NA in really healthy subjects

No explanation can be offered for the very high levels noted in 4 cases, i e 2 5 to 16 4 μg NA/l These high values may be due to mental disease, or to the clinical treatment they were receiving The main source of this NA may be the brain tissue, but very high plasma NA values have also been found in *e g* schizophrenics receiving chlorpromazine (Carlsson *et al* 1967)

The results obtained, and some preliminary histochemical and biochemical results showing increased catecholamine levels in rabbit brain after i v or i p administration of NA and DA after pretreatment with reserpine and MAO inhibitors (Dahlstrom and Haggendal, unpublished results) suggest that the blood-CSF/brain or blood/brain barriers are permeable to catecholamines at some conditions

However, the animals in these experiments were given large amounts of NA and DA and the results may be due to secondary effects of the catecholamines After blood/brain barrier lesions catecholamines can penetrate into the rabbit brain parenchyma (Hamberger and Hamberger 1966)

It seems that some acid monoamine metabolites, *e g* homovanillic acid, can be actively transported from the brain and that this transport can be prevented by probenecid (Werdinius 1966) The origin of the monoamines and their metabolites in the CSF appears to require further investigation and the present findings that some metabolites such as conjugated NA can pass through the blood/CSF barrier appears to be of importance for the discussion of the problem

The work was supported by the Medical Faculty of Göteborg the Swedish State Medical Research Council (14X—166—02 and B67 14X—166—03), the AB Leo Fund for Research and the Pfannenstill Foundation

For technical assistance we are most indebted to Miss Monica Ericsson

References

ANDÉN N E A CARLSSON N A HILLARP and T MAGNUSSON Noradrenaline release by nerve stimulation of the spinal cord *Life Sci* 1965 *4* 129—132

AXELROD J O methylation of epinephrine and other catechols in vitro and vivo *Science* 1957 *126* 400—401

AXELROD J Metabolism of epinephrine and other sympathomimetic amines *Physiol Rev* 1959 *39* 751 7 0

CARLSSON A and B WALDECK A fluorimetric method for the determination of dopamine (3 hydroxytyramine *Acta physiol scand* 1958 *44* 293—298

CARLSSON A and M LINDQVIST In vivo decarboxylation of α-methyl DOPA and α methyl metatyrosine *Acta physiol scand* 1962 *54* 87 94

CARLSSON C S J DENCKER G GRIMBY and J HAGGENDAL Noradrenaline in human blood plasma and urine during exercise in patients receiving large doses of chlorpromazine *Acta pharmacol*

HAGGENDAL J An improved method for fluorimetric determination of small amounts of adrenaline and noradrenaline in plasma and tissues *Acta physiol scand* 1963a *59* 242—254

HAGGENDAL J The presence of conjugated adrenaline and noradrenaline in human blood plasma *Acta physiol scand* 1963b *59* 255 260

HAGGENDAL J Catecholamines in human cerebrospinal fluid Presented at the XII Congress of Physiology Turku 1966 *Acta physiol scand* 1966 *68* Suppl 277 84

ROTHBALLER, A B, The effects of catecholamines on the central nervous system Symposium on catecholamines held on Oct 16—18, 1958 at the National Institutes of Health, Bethesda Maryland Ed O Krayer, The Williams and Wilkins Company 1959 494—547

SCHAIN, R S, Neurohumers and other pharmacologically active substances in cerebrospinal fluid A review of the literature *Yale Biol Med* 1960 33 15—36

VALK, A de T Jr and H L PRICE The chemical estimation of epinephrine and norepinephrine in human and canine plasma I A critique of the ethylenediamine condensation method *J clin Invest* 1956 35 837—841

WEIL MALHERBE, H and D W LIDDELL, Adrenaline and noradrenaline in cerebrospinal fluid *J neurol Neurosurg Psychiat* 1954 17 247—249

WERDINIUS B, Effect of probenecid on the level of homovanillic acid in the corpus striatum *J Pharm Pharmacol* 1966 (in press)

Acta physiol. scand. 1967. 69. 147—152

From the Department of Pharmacology, University of Goteborg, Sweden

The Effect of High Pressure Air or Oxygen with and without Carbon Dioxide Added on the Catecholamine Levels of Rat Brain

By

JAN HÄGGENDAL

Received 14 September 1966

Abstract

HÄGGENDAL, J., *The effect of high pressure air or oxygen with and without carbon dioxide added on the catecholamine levels of rat brain.* Acta physiol. scand. 1967. *69.* 147—152

cholamine or monoamine containing neurons in the brain

In most types of diving and also in the therapy of some ischemic or infectious diseases the body may be exposed to gas mixtures at high pressure. Increased partial pressure of the components in the gas mixtures may give typical symptoms from the central nervous system (CNS). Oxygen at high pressure has clearcut action on CNS with nervous sensations as restlessness, and motor effects as muscle twitching and even convulsions. Air at high pressure has a narcotic effect often discussed as being due to the nitrogen but the carbon dioxide appears also to be of importance (e.g. Bean 1945, 1950, 1965, Bennet 1966). Adolfson (1964, 1965 a and b) and Adolfson and Muren (1965) found that hyperbaric air intoxication in man at 13 (ata) had clearcut symptoms which were much more pronounced than at 10 ata. There were changes in mood and affectivity with euphoria. Some changes in perception were reported showing several similarities with effects of hallucinogenic drugs. There was also a more or less severe impairment of consciousness. The motor functions were affected

with a general clumsiness of the fine movements and often with a "stiffness" or slight paralysis of the facial muscles

After O_2 at high pressure changes in different endocrine organs have been found Bean (1945 a), Troell (1947), Bean and Johnson (1954), and Edström and Rockert (1962) found a stimulating action on the sympathetic nervous system and the adrenals Edstrom and Rockert (1962) found after O_2 at high pressure adrenal hypertrophy, decreased weight of the thymus, and increased weight of the thyroid and Sjostrand (1964) found increased uptake of I^{131}

In the CNS, however, it has been difficult to observe any consistent changes after O_2 at high pressure Histologically Edstrom and Rockert (1962) did not find demyelination or other degenerative signs in CNS In fact, several authors have been inclined to consider the action of O_2 at high pressure on CNS to be entirely functional (Bean 1945 a and b for ref) However, Bean (1945 b) found lesions in CNS and also recently selective CNS lesions have been reported after repeated exposures to O_2 at high pressure (Balentine and Gutsche 1966)

The catecholamines noradrenaline (NA) and dopamine (DA) in CNS are probably transmitters in neurons which are of importance for some mental functions, and for muscular coordination and also for sympathetic functions, see Hillarp *et al* (1966) For instance most if not all psychopharmaca have effects on the catecholamines in CNS Histochemically DA containing neurons have been mapped out being of importance for the muscular coordination Catecholaminergic neurons in hypothalamus appear to be of importance for sympathetic and some endocrine functions

The parallelism between the reported effects on O_2 and air at high pressure and different agents affecting the catecholamine metabolism made it of interest to study the catecholamine levels in rat brain after exposure to O_2 and air at high pressure

Methods

Hooded rats from a strain bred in this laboratory weighing about 200 g were treated with different gas mixtures in a pressure chamber which had a volume of 35 l and was provided with an inspection window The different gases were pure O_2 O_2 and 1 % CO_2 air and air and 1 % CO_2 Before the pressure was increased the chamber was washed with the actual gas and also ventilated during the exposure to diminish the accumulation of CO_2 The temperature in the chamber was about 25 °C As a rule 3 rats were exposed at the same time After the exposure the pressure was rapidly reduced The whole brains were immediately dissected out put in 0 4 N perchloric acid one brain per sample and homogenized After purification of the extracts on columns of strong cation exchange resin (Dowex 50 W X8) the NA and DA were estimated fluorimetrically in an Aminco-Bowman spectrophotofluorometer after oxidation and rearrangement according to the principles of the trihydroxyindole reaction NA was estimated according to Bertler, Carlsson and Rosengren (1958) and Haggendal (1963) and DA according to Carlsson and Waldeck (1958) modified by Carlsson and Lindqvist (1962 Control brains from as a rule 2 3 untreated rats were assayed parallelly at every experimental occasion

Results

The NA and DA brain levels (mean ± S E M) in per cent of the mean values of the group of normal rats determined at every experimental occasion, the exposure time, and the exposure pressure of the different gas mixtures are given in Fig 1 and 2

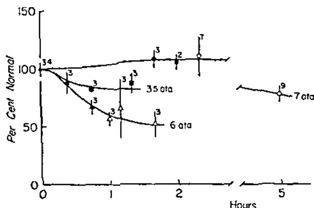


Fig 1 The noradrenaline (NA) levels in rat brain after high pressure exposure to air (○), air and 1 % CO_2 (●), O_2 (△), and O_2 and 1 % CO_2 (▲) at 6 to 7 ata, and O_2 and 1 % CO_2 at 3 5 ata (□) The exposure pressure for the NA value after 2 hrs was 4 ata The NA levels (mean ± S E M, n = number of observations) in per cent of the mean value from control rats assayed simultaneously Mean value of all the control rat was 0 33 ± 0 021 μg g n = 34

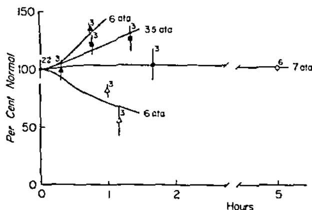


Fig 2 The dopamine (DA) levels in rat brain after high pressure exposure to air (○) air and 1 % CO_2 (●) O_2 (△), and O_2 and 1 % CO_2 (▲) at 3 5 to 7 ata The DA levels (mean ± S E M n = number of observations) in per cent of the mean value from control rats assayed simultaneously Mean value of all the control rats was 0 67 ± 0 069 μg/g n 22

After exposure to air up to 7 ata for about 5 hrs the NA and DA levels were normal with a tendency towards decrease for the NA at the longer exposure time The difference, however, between the exposured and the untreated animals was not statistically significant No convulsions appeared and the animals behaved in gross like the control rats

After exposure to air and CO_2 at 4 and 7 ata for about 2 hrs the catecholamine levels appeared to be normal The respiratory frequency was increased at the exposure No convulsions were seen in the five exposure rats but one showed after about 20 min of exposure a tendency to motor incoordination

After pure O_2 at 6 ata for 1 hr to 1 hr 40 min the NA levels were decreased to about 55 per cent of the normal levels The NA values at three experimental occasions calculated as one group differed significantly ($P < 0.005$) from the values of the corresponding control animals The DA levels were also less than normal Eight of the nine observed animals had convulsions or were in bad condition after about 1 hr The onset of the abnormal behaviour varied, however for the animals from about 1/2 to more than 1 hr after the beginning of the exposure

After O_2 and CO_2 exposure at 6 ata the NA levels were 87 per cent after 19 min and 66 per cent after 45 min The DA levels were 98 and 134 per cent, respectively The respiratory frequency was increased and all the six observed animals had convulsions after about 15 to 20 min Thus the convulsions appeared earlier at the same pressure when CO_2 was added to O_2

When the rats were exposed to O_2 and CO_2 mixture at 3 5 ata the levels of NA and DA were about 85 and 120 per cent, respectively No convulsions were seen in the six observed rats From the obtained results it thus seems as the DA levels were increased after addition of CO_2 to the O_2, while the levels were decreased after pure O_2

A few of the rats with convulsions died in the pressure chamber during the exposure The amine levels of these rats did not markedly differ from the levels of the other rats in the group

Discussion

Since in man exposed to air at high pressure the disturbance of mental and psycho motor functions were rather diffuse and uncertain up to less than 10 ata (Adolfson and Muren 1965), it is of interest to see that after exposure to air the NA and DA levels in rat were about normal except for the tendency to decreased NA levels after long time (about 5 hrs) at high pressure (7 ata) A study of the catecholamine levels after exposure to air at more than 10 ata might thus be of interest

The effect of O_2 is much more pronounced and appears at lower pressures in man and animals There is considerable individual variations in susceptibility and an absolutely safe threshold is difficult to establish But at rest about 3 ata for about 1 hr has been dicussed as an approximate threshold (see Bein 1965 for review) In the present investigation the decrease of the NA levels appeared to be more evident after higher O_2 pressure and longer exposure time This may be compared to the well established fact that the higher ambient pressure the more intense are the defects in mental and psychomotor performance

The addition of CO_2 appeared to have no effect on the NA decrease caused by pure O_2 The material, however, is small and further studies are indicated The same seems to be true for the increase of the DA levels found after CO_2 addition Pure O_2 gave decreased DA levels However, it must be observed that these two groups after treatment during 45 min or longer did not differ statistically significant The fact that the addition of CO_2 to the O_2 at 6 ata caused an earlier onset of the convulsions illustrates that the CO_2 tension is of importance for the development of 'O_2-intoxication"

It may be possible that the observed changes of the NA and DA levels were caused by the convulsions However, the NA and DA levels appeared to be changed after exposure to O_2 and CO_2 at 3 5 ata without any convulsions being present There was also a tendency towards decreased levels after long term treatment (about 5 hrs) with air which gave no convulsions After convulsions caused by electrochock or some drugs as *e g* pentylenetetrazol increased 5-hydroxytryptamine (5-HT) levels

in brain have been observed by many investigators (see *e g* Bertaccini 1959 for results and lit) The NA levels appear to have been inverstigated more seldom However, Breitner, Picchioni and Chin (1964) found increased 5 HT and decreased NA levels, but Engel, Hanson and Strombergsson (preliminary results, this laboratory 1966) showed increased 5 HT and increased or normal NA levels after electroshock with convulsions

By daily repeated short exposures animals could be treated with O_2 at high pressure for weeks with only a low percentage of convulsions (see Edstrom and Rockert 1962 for results and lit) It may be of interest to study the brain levels of NA, DA and 5 HT in animals of this type

The present investigation indicates that at exposure to certain gases at high pressure catecholamine containing neurons in brain may be affected Some symptoms at the exposure may be due to the effect of the gases on catecholamine (possibly also 5-HT) containing neurons in CNS It seems also to be possible biochemically to evaluate different gas mixtures at high pressure with respect to the catecholamine neurons in CNS

The work was supported by a grant from the Swedish State Medical Research Council (Y 379) My thanks are due to Dr L Troell Surgeon General of the Navy for the disposal of the pressure chamber For technical assistance I am indebted to Miss Monica Ericsson

References

ADOLFSON J *Compressed air narcosis* A study of human behaviour at increased ambient pressure From the Institution of Psychology University of Goteborg and the Office of Surgeon General Naval Staff R S N Stockholm Sweden 1964

ADOLFSON J Deterioration of mental and motor functions in hyperbaric air *Scand J Psychol* 1965 a 26—3?

ADOLFSON J *Psychotic symptoms in hyperbaric air* Presented at the 2nd int Symp Underwater Medicine Ustica Italy Sept 1965 b

ADOLFSON J and A MUREN Air breathing at 13 atmospheres Psychological and physiological observations *Forsvarsmedicin* 1965 *1* 31—37

BALENTINE J D and B B GUTSCHE Central nervous system lesions in rats exposed to oxygen at high pressure *Amer J Path* 1966 *48* No 1 107—127

BEAN J W Effects of oxygen at increased pressure *Physiol Rev* 1945 a *25* 1—147

BEAN J W Alterations in C N S associated with chronic motor disabilities induced by O_2 at high pressure *Proc Soc exp Biol* (N Y) 1945 b *58* 20—21

BEAN J W Tensional changes of alveolar gas in reactions to rapid compression and decompression [illegible]

BE [illegible]

BE [illegible]

[illegible] 410—414

BENNET P B The aetiology of compressed air intoxication and inert gas narcosis Pergamon Press London 196?

BERTACCINI G Effect of convulsant treatment on the 5-hydroxytryptamine content of brain and other tissues of the rat *J Neurochem* 1959 *4* 21? —222

BERTLER A A CARLSSON and E ROSENGREN A method for the fluorimetric determination of adrenaline and noradrenaline in tissues *Acta physiol scand* 1958 *44* 273—292

BREITNER C A PICCHIONI and L CHIN Neurohormone levels in brain after CNS stimulation including electrotherapy *J Neuropsych at* 1964 *5* No. 3

CARLSSON A and M LINDQVIST In vivo decarboxylation of α methyl metatyrosine *Acta physiol scand* 19?2 *54* 87—94

CARLSSON A and B WALDECK A fluorimetric method for the determination of dopamine (hydroxytyramine *Acta physiol scand* 19[illegible]

Edstrom J E and H Rockert, The effect of oxygen at high pressure on the histology of the central nervous system and sympathetic and endocrine cells *Acta physiol scand* 1962 *55* 255—263

scand 1964 62 94—100

Troell, L, Studies on experimentally provoked ileus with references to inhalational therapy *Acta chir scand* 1947 *95* Suppl 122

Acta physiol scand 1967 69 153—157

From the Department of Histology Karolinska Institutet Stockholm and the Department of Pharmacology University of Göteborg Sweden

Studies on the Transport and Life-span of Amine Storage Granules in the Adrenergic Neuron System of the Rabbit Sciatic Nerve

By

ANNICA DAHLSTRÖM and JAN HÄGGENDAL

Received 15 September 1966

Abstract

DAHLSTRÖM A and J HÄGGENDAL *Studies on the transport and life span of amine storage granules in the adrenergic neuron system of the rabbit sciatic nerve* Acta physiol scand 1967 69 153—157

Studies have earlier been made on the transport of noradrenaline (NA) granules in the sciatic nerve of rat and cat (Dahlström and Häggendal 1966) The figures for the rate of transport were about 5 mm per hr for the rat and about 10 mm per hr for the cat The life span of the granules in the adrenergic terminal system of the sciatic nerves was calculated to be about 35 days for the rat and about 70 days for the cat Because of these species differences it was of interest to study also the rabbit

The present study on the transport and life span of the amine storage granules of the rabbit has been performed in the same manner as earlier used for rat and cat The accumulation of NA above a ligation of the sciatic nerve after different time periods was quantified and the content of NA in the terminals belonging to this neuron system was estimated by means of denervation experiments

Material and methods

All [illegible] rabbits of both sexes weighing between [illegible] kg were used

For the study of the NA accumulation above a nerve ligation 10 rabbits were used [illegible] operated and [illegible] under Nembutal® anesthesia [illegible] mg/kg b w the sciatic nerve was compressed bilaterally using a metal rod and a silk suture as described earlier (Dahlström 19[illegible]) [illegible] compression a silk ligature was [illegible] at the site of the lesion in order [illegible] NA [illegible] the site of compression during the accumulation time The animals were [illegible]

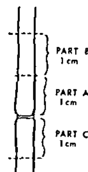


Fig 1 Schematic illustration of the dissection of the rabbit sciatic nerve after a ligation

at 12 and 24 hrs after the operation and the nerves were dissected out The NA content in different parts of the nerve (Fig 1) was estimated The 1 cm proximal to the ligation (part A), the 1 cm just above A (part B) and the 1 cm just below the ligature (part C) were estimated separately,

tissue was deep frozen and masticated together with dry ice in an ordinary meat grinder (Husqvarna no 5), put into cold PCA (0 4 N) and homogenized (Ultra-Turrax) The amount of PCA used (in m
alway
tion
X 4)
gren (1958)

Two normal rabbits were also treated in the same way as described above To the PCA sample of one leg of each rabbit a known amount of NA (20—25 μg) was added before the homogenization to check the recovery of NA

Results

As seen from Table I the amount of NA in 1 cm of a normal rabbit nerve was found to be 10 1 ± 2 48 ng (mean ± S E M) After ligation the amount in part A increased along a straight line during the time interval investigated (Fig 2) At 12 hrs the amount of accumulated NA was 38 9 ± 2 35 ng (mean ± S E M) The amount of NA in 1 cm of the sciatic nerve had thus increased approximately 4-fold In part B, just above part A, no significant change in the NA levels was observed at 12 h The part below the constriction (part C), however, showed slightly decreased levels (Table I)

The NA contents of the terminal system emanating from the sciatic nerve, expressed as the difference between the total NA contents in normal and denervated

TABLE I The noradrenaline (NA) content in ng per cm of the rabbit sciatic nerve after bilateral ligation The values are corrected for the recovery of added NA Dissection of the nerve was performed according to Fig 1

Sample	Recovery per cent	Hours after ligation of the sciatic nerve 0 hr	12 hrs	24 hrs
Part A (Fig 1),	58	20 5	45 0	49 0
the 1 cm prox to		*14 2*	*33 0*	*83 4*
the ligation			42 5	
	68	8 1	31 1	62 0
		7 5	38 7	
		5 0	42 9	
		5 6		
Mean ± S E M		10 1 ± 2 48	38 9 ± 2 35	64 8 ± 6 24
Part B (Fig 1)	68		8 1	
the 1 cm prox to part A			5 7	
Mean ± S E M			6 9 ± 1 20	
Part C (Fig 1)	58		8 2	
the 1 cm below			2 4	
the ligation	68		2 5	
			5 1	
Mean ± S E M			4 6 ± 1 37	

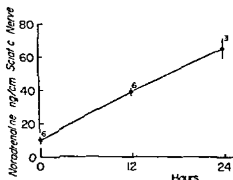


Fig 2 The amount of noradrenaline in ng (Mean ± S E M) in the 1 cm part of the rabbit sciatic nerve just proximal to a ligation

legs were found to be 3 12 ± 0 27 μg The loss of NA in the denervated leg was about 47 per cent of the NA of the control legs, i e about the same amount as the one obtained earlier for both rat and cat (38 per cent Dahlström and Häggendal 1966)

TABLE II. The effect of unilateral ligation and cutting (denervation) of the sciatic nerve on the NA content of the hind leg of the rabbit. The values are corrected for the recovery of added NA and for 20 per cent post mortem loss during the preparation

Rabbit no.	Body weight (*kg*)	*Sex*	Time after *denervation*	Recovery per cent *of added NA*	Difference of NA in *μg between control and denervated leg*
1	1.8	♂	4 weeks	95	3.0
2	1.9	♀	4 weeks		2.55
3	1.7	♂	3 weeks	87	4.17
4	2.0	♂	3 weeks		2.75
				Mean ± S.E.M.	3.12 ± 0.27

	Weight of soft tissue in g per leg. Mean ± S.E.M.	*NA content in μg per leg. Mean ± S.E.M.*
Denervated leg	139.1 ± 6.40 n = 4	3.53 ± 0.78
Control (contralateral) leg	164.0 ± 9.44 n = 4	6.65 ± 0.67
Normal leg (2 individual rabbits)	154.5 ± 7.24 n = 2	6.42 ± 0.60

Discussion

The sensitivity of the biochemical assay method for catecholamines is high and has been discussed earlier (Bertler *et al.* 1958, Häggendal 1963). The recovery of added NA was corrected for. For the hind leg samples corrections for postmortal destruction of 20 per cent (Sedvall 1964) were made, since it was supposed that the rate of destruction of NA in skeletal muscle is about the same in rabbit as in cat.

The accumulation of NA occurred rather rapidly (Fig. 2) but slower than in both rat and cat (Dahlström and Häggendal 1966). The possibilities that this accumulation might be due to a local synthesis of the amine or the amine granules, have been discussed in the above mentioned paper and found to be less likely. The possibility that the NA might be located free in the axoplasm was also discussed and several facts supporting the view that the accumulated NA is stored within amine granules were given. The theory that the accumulation of NA is due to a piling up of amine granules synthetized in the nerve cell bodies and transported via the axons to the terminals seems to be valid (Dahlström 1965, Dahlström, Fuxe and Hillarp 1965, Dahlström and Häggendal 1966). The course of accumulation thus most certainly reflects the normal rate of transport (Dahlström and Häggendal 1966).

Since no sign of NA accumulation was found in part B and no biochemical sign of a retrograde accumulation (Dahlström 1965) was observed (Table I), the calculations on the rate of transport have been performed only using part A. As in the

earlier study on rat and cat the amount of NA in the sciatic nerve and also in the adrenergic terminals has been presumed to be proportional to a certain number of amine storage granules

On the basis of the assumptions earlier discussed the rate of transport of amine granules was calculated The normal sciatic nerve contained about 10 ng per cm After 12 hrs the amount above the constriction had increased to about 40 (38 9) ng, which means that about 30 (28 8) ng, corresponding to 3 $\left(\frac{30}{10}\right)$ cm normal nerve had been transported to the distal cm The rate of transport was thus about 3 $\left(\frac{30}{12}\right)$ mm per h

The decreased amount of NA in the hind legs after cutting the sciatic nerve most probably represents the NA content of the adrenergic terminals belonging to the NA nerve fibres in the sciatic nerve The rate of transport of amine granules in all probability reflects the rate of destruction in the terminals, since a change in the amount of granules, *e g* an overfilling, would otherwise be the result Thus, if the terminals contain granules corresponding to 3 12 μg of NA, and 30 ng of granule-bound NA was transported down per 12 hrs, the life span of the granules would be $\left(\frac{3120}{30 \times 2}\right)$ approximately 50 days

Like the figures of transport and life span obtained for rat and cat (Dahlstrom and Haggendal 1966) the figures obtained in this study for the albino rabbit must be considered as approximate However, they show that there exist differences in both rate of transport and life span of the granules in different species, but that the differences seem to be rather small, staying within the same order of magnitude

This study was supported by the Swedish Medical Research Council (NB 14X 166-02 and B67 14X 166-03

For technical assistance we are most indebted to miss Agneta Wilen

References

BERTLER A, A CARLSSON and E ROSENGREN A method for the fluorimetric determination of adrenaline and noradrenaline in tissues *Acta physiol scand* 1958 *44* 273—292

DAHLSTROM A Observations on the accumulation of noradrenaline in the proximal and distal parts of peripheral adrenergic nerves after compression *J Anat* Lond 1965 *99* 677—689

DAHLSTROM A, K FUXE and N A HILLARP Site of action of reserpine *Acta Pharmacol* (Kbh) 1965 *22* 277—292

DAHLSTROM A and J HAGGENDAL St[illegible]
nules in a peripher[illegible]

HAGGENDAL J An i[illegible]
and noradrenaline [illegible]

SEDVALL G Noradrenaline storage in skeletal muscle *Acta physiol scand* 1964 *60* 39—50

Acta physiol scand 1967 69. 158—166

From the Department of Histology Karolinska Institutet Stockholm and the Department of Pharmacology, University of Goteborg Sweden

The Transport of Noradrenaline Between two Simultaneously Performed Ligations of the Sciatic Nerves of Rat and Cat

By

ANNICA DAHLSTROM

Received 16 September 1966

Abstract

DAHLSTROM, A, *The transport of noradrenaline between two simultaneously performed ligations of the sciatic nerves of rat and cat* Acta physiol scand 1967 *69* 158—166

The behaviour of noradrenaline (NA) in double ligated rat and cat sciatic nerves was studied histochemically, using the fluorescence method of Hillarp and coworkers and biochemically using the trihydroxyindole method It was found that a large amount of the NA in the nerve between the two ligations was transported down to the part just above the low ligation By both methods it was shown that during the first hours after the ligation the course of accumulation of NA in the nerve just proximal to the low ligation was about the same as the accumulation curve for the nerve (connected to the cell bodies) above the high ligature Thereafter no further changes were found to occur in the nerve between the constrictions whereas in the part above the high ligature the accumulation of NA continued as described earlier The total NA content in the nerve between the two ligatures did not exceed the amount of a normal nerve of the same length It was concluded that the transport mechanism in the adrenergic fibres is mainly independent of the cell body (and the nerve terminals) and that no increase in the NA content in the axons occurs after a ligation of the nerve Furthermore the present study gives support to the earlier obtained data for the transport of amine granules in the sciatic nerve of rat and cat

The behaviour of noradrenaline (NA), in nerves after axotomy or ligation has been studied (Dahlstrom and Fuxe 1964 a, b, Dahlstrom 1965) with the use of the histochemical fluorescence method of Hillarp and co workers (for references, see *e g* Hillarp Fuxe and Dahlstrom 1965) It was found that above a constriction there rapidly occurs an accumulation of NA in bulgy and gradually distorted *axons* Also, a very small accumulation distal to the ligation could be observed (*Dahlstrom* 1965) These observations have been presumed to represent signs of a large proximo-distal flow (transport) of the transmitter, in accordance to the theory of Weiss (1961), and as signs of a much smaller retrograde flow, like the one reported by Lubin'ska *et al* (1963) for acetylcholinesterase (AcChE) Certain observations, *e g* the disappearance of the accumulated NA after reserpine treatment (Dahlström 1965), suggested that the accumulated NA in the axons, as the NA in normal

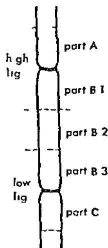


Fig 1 Schematic illustration of the dissection of the double ligated cat sciatic nerve prior to the assay of the NA distribution

adrenergic nerves (Carlsson 1965, Malmfors 1965), was stored within particles (see Discussion, Dahlstrom 1965), the so called amine storage granules (Euler and Hillarp 1956)

Biochemical studies on the accumulation of NA after ligation of the sciatic nerve in rat and cat (Dahlstrom and Haggendal 1966) showed that the amount of NA found above the lesion increased approximately linearly during at least 24 hrs Because of the complex protein structure of the amine granules (Hillarp 1960) within which the transmitter is stored, these storage granules are probably mainly — if not entirely — manufactured in the cell body of the neurons where high concentrations of RNA exist (Hyden 1960) The axons contain only very low concentrations (Koenig 1965) The NA granules would thus be transported through the axons down to the terminals where they are stored in the varicosities (see *i a* Norberg and Hamberger 1964 Malmfors 1965) Using the biochemical data obtained the effective rate of this transport of NA (granule bound) was calculated to be about 5 mm/h for the rat and about 10 mm/h for the cat (Dahlstrom and Haggendal 1966)

The down transport of the granules is in all probability mainly due to an active transport as suggested by Weiss (1961) and Ochs (1963) Preliminary histochemical studies on rat sciatic nerve with 2 simultaneously performed constrictions showed that an accumulation of amines occurred also in the nerve part just above the low ligature (Dahlstrom 1965), while it was found by biochemical analysis of the nerve part between the two ligations that the total amount of NA within this part was unchanged for at least 18 hrs in the rat and for 12 hrs in the cat (Dahlstrom and Haggendal 1966) A redistribution of the NA within the nerve part seemed to have occurred in this part of nerve, separated from both the cell bodies and the

The purpose of the present investigation was to make a closer

of the transport of NA in double ligated sciatic nerves and to

changes in the distribution of NA in the separated nerve part



Fig 2 Histochemical demonstration of the accumulation of NA in double ligated sciatic

...

tion, high ligature Large accumulations of NA in swollen and bulgy nerve fibres can be seen above the ligation This accumulation is consid

...

lations of fluorescent material in moderately swollen axons can be observed This accumulation is of about the same size as those present in a) and b) and much smaller than that observed in c) above the high ligature Below the ligature is indicated nerve fibres with retrograde accumulations (⟼) which are somewhat more developed than the retrograde accumulations observed in a) and b)

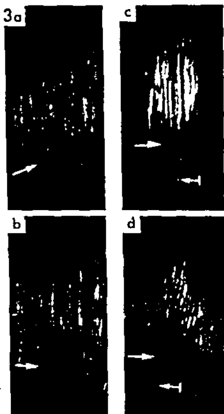
Fluorescence microphotograph × 28

Material and methods

Ten cats of both sexes weighing between 1 8—2 9 kg, and 26 rats (male albino of the Sprague-Dawley strain) weighing between 200—2.0 g were used Both sciatic nerves were compressed

...

low ligature were dissected as shown in Fig 1 The middle part was divided into 3 pieces (... B 2 and B 3), and NA determined in single or on some occasions in two pieces After the dissection the nerve pieces (all of 1 cm length were immediately put into ice-cooled 0 4 N perchloric acid (7 ml) with 20 mg of ethylene-diamine tetraacetate (EDTA) added After homogenization, using an Ultra-Turrax (Janke & Kunkel homogenizer and centrifugation, the extracts were purified on columns of strong cation exchange resins Dowex W 50 X 8 NA was determined in an Aminco-Bowman spectrophotofluorometer In every experiment at least 2 samples containing normal nerves of 0 5 cm length with an addition of known amounts of NA (50 or 100 ng) were included in the series in order to check the recovery through the estimation procedures



The ligation is indicated (⟶) a) 1 hr after operation, high ligature Green fluorescent material

tion Below the ligation is indicated retrograde accumulations with very week fluorescence intensity (⟼)

low the ligature with very weakly fluorescent retrograde accumulations are observed (⟼)
Flurorescence microphotograph × 28

Results

Histochemical analysis RAT At 1 and 2 hrs after ligation the middle part of the nerve between the ligations (part B, Fig 1) contained a few visible, weekly fluorescent nerve fibres Above the high ligature and also above the low ligature accumulations of fluorescent material could clearly be observed lying within moderately swollen, bulgy nerve fibres The accumulations had the same appearance in both parts, regarding both intensity and amount of fluorescent material Fig 2 a and b) Below the low ligature a small retrograde accumulation (Dahlström 1965) could be seen in the 0 5 mm just distal to the ligation Also below the high ligature signs of retrograde accumulation could be seen, but usually with a fainter fluorescence (Fig 2 a and b) At 3, 4 and 6 hrs the accumulation in the part above the low ligature was fully developed (Fig 2 d and did not change in appearance during the following 6 hrs Nor was any change observed in the retrograde accumulation below the high constriction The accumulation above the high ligature however, increased gradually in amount and i g h Fig 2 c) during the time interval investigated as described earlier (Dahlström 1965 Dahlström and Häggendal 1966) Likewise could a small increase be observed to occur in the retrograde accumulation below the low constriction (Fig 2 d At 12 hrs no or very little specific fluor

TABLE I The NA content in the sciatic nerve of cat after two simultaneously performed ligations The dissection was performed as described in Fig 1 The amount of NA (ng) per 1 cm nerve is given if not otherwise noted

Time after ligation	Part A	Part B 1	Part B 2	Part B 3	Part C	∑B Parts
1 hr	10 5	8 1	4 7	12 6		25 4
	14 5	9 0	5 4	9 1		23 5
	17 2	2 6	5 5	8 0	5 9	16 1
	15 0	6 9	6 5	12 3		25 7
Mean ± S E M	14 3 ± 1 48	6 65 ± 1 40	5 52 ± 0 56	10 5 ± 1 11		
2 hrs	15 9	5 0	7 4	17 0	6 1	29 4
	15 5			15 2		29 6
	11 8	6 5	6 5	13 0	6 7	26 0
	14 9			13 7		26 7
Mean ± S E M	14 5 ± 0 88	5 75 ± 0 75	6 95 + 0 45	14 7 ± 0 80	6 4 ± 0 30	
3 hrs	37 0	6 5	2 3	16 3	12 5	25 1
	15 4	10 8	6 8	14 8	8 5	32 2
Mean ± S E M	21 2 ± 5 80	8 6 ± 2 15	4 5 ± 1 25	15 5 ± 0 75	10 5 ± 2 0	
	19 0	7 1 } 1 5 cm	13 0 } 1 5 cm	25 0 } 1 5 cm		46 1 } 4 5 cm
	20 7	2 8 }	8 0 }	22 2 }		33 0 }
Mean ± S E M	19 8 ± 0 85	4 9 ± 2 15	10 5 ± 2 50	23 6 ± 1 40		39 5 ± 6 55
				Content of 4 5 cm normal nerve 38 7 ng		
6 hrs	63 9	6 2	4 3	17 9	10 1	28 4
	56 0	2 2	7 8	13 5	7 3	23 5
Mean ± S E M	59 9 ± 3 95	4 2 ± 2 0	6 0 ± 1 75	15 7 ± 2 20	8 7 ± 1 40	
					Mean ± S E M	26 96 ± 1 09

Normal value 9 2 ng cm 0 63 (Dahlstrom and Häggendal 1966)

Content of 3 cm normal nerve 27 6 ng

within adrenergic axons could be seen in the middle part (corresponding to part B 2 in Fig 1) of the nerve between the two ligations A few adrenergically innervated vessels were usually observed at all times after ligation as in normal animals, and sometimes such vessels were seen passing through the site of ligation, apparently undamaged by the procedure

CAT At 1 hr after the operation the accumulation of fluorescent material above the two ligatures had about the same appearance regarding both fluorescence intensity and length (Fig 3 a and b) The middle piece of the part between the two constrictions contained a few weakly green fluorescent fibres with thin enlargements at irregular intervals, like those often observed in normal sciatic nerves of rat and cat (see *e g* Dahlstrom and Fuxe 1964 b, and unpubl obs) Retrograde accumula-

tions of 0 2—0 5 mm length could be seen below both constrictions, but with very low fluorescence intensities

Three hours after the ligation the accumulation of fluorescent material above the high ligature was observed to be larger than that above the low ligature (Fig 3 c and d) The intensity of the fluorescence was increased, and the axons were more swollen and bulgy and the length of the distorted axons was generally larger The accumulation above the low ligature was seen to be somewhat larger than that observed at 1 hr above the ligature (see Fig 3 a, b and d) A few weakly fluorescent fibres of normal appearance were observed in the middle piece (corresponding to part B 2, Fig 1) of the nerve between the constrictions, and small enlargements could still be observed The retrograde accumulation below the ligatures was at 3 hrs clearly visible but no obvious difference could be observed between the retrograde accumulation below the high and the low ligature

NA assay CAT The recovery per cent through the estimation procedures varied between 66 and 85 in the different experiments The NA recovery in the two addition samples in each experiment never differed from each other more than 6—10 per cent A mean of the two values was therefore used to correct the obtained figures for recovery

In the part proximal to the high ligature (part A, Fig 1) the accumulation increased from 0—6 hrs as described earlier (Dahlstrom and Haggendal 1966) In part B 3 (Fig 1), just proximal to the low ligation, the accumulation of NA followed about the same course as in part A (Table I) for the first 2 hrs Thereafter no further increase occurred and the amount was essentially unchanged up to 6 hrs

The NA content in the 2 proximal parts (B 1 and B 2) of the middle nerve part gradually decreased from normal values of about 9 ng per cm (Dahlstrom and Haggendal 1966) *down to about 4 (part B 1) and 6 ng (part B 2) at 6 hrs* The onset of the decrease could be observed already 1 hr after the ligation (Table I) In the C-part, below the low ligature, no certain changes could be observed during the time studied

At all estimations the sum of the NA content in all B parts of each sciatic nerve was about the same as in a normal unligated nerve of the same length (Table I)

In one cat the ligations were placed at an interval of 4 5 cm All B parts were of 1 5 cm length and dissected out 3 hrs after operation The changes in NA content in the B parts followed the pattern described above and the sum of the NA content in all B parts was about the same as in a normal sciatic nerve of 4 5 cm length (Table I)

Discussion

The specificity of the histochemical fluorescence method used has been discussed previously (see *i a* Corrodi Hillarp and Jonsson 1965) The accumulating material, developing a strong green fluorescence after formaldehyde treatment, has the properties of a catecholamine, which spectrophotofluorimetrically has been identified as NA (Dahlstrom and Haggendal 1966)

The results obtained in the present study, and particularly the observation that the accumulation of NA for the first 2 hrs after the operation was about the same for the low B part above the low ligature as for the A-part above the high ligature both histochemically and by assay of NA, indicate that the mechanism for convection or transport of the amine granules was in main the same whether the axons were connected to the cell bodies or not Thus, a pumping mechanism originating in and dependent on the cell body may be excluded Streaming within the axoplasm, like the one observed in the cytoplasm of certain algae (see *i a* Hayashi 1964), or some kind of peristaltic activity (as proposed by Weiss 1961 and Ochs 1963) in the axon wall, represent two possible explanations to the obtained results Of these the latter one seems to be the most probable because of the bulgy and distorted appearance seen in the accumulated fibres, as pointed out by Weiss (1961) Certain movements of the axon wall has also been observed in microcinematographic studies of peripheral nerves (Weiss, Taylor and Pillai 1962) Furthermore, recent *in vitro* studies on Schwann cells, which are *in vivo* enclosing the entire adrenergic nerve fibre, have revealed pulsatile activities at varying contraction rates (Ernyei and Young 1966) The Schwann cells *in vivo* might thus serve to drive the axoplasm in a proximo distal direction as also proposed by these authors

The figure of the rate of transport of NA granules in the sciatic nerve obtained in an earlier study (Dahlström and Häggendal 1966) was found to be about 10 mm per hr for the cat and about 5 mm for the rat As found by assay a large part of the NA in the cat sciatic nerve between the ligations (3 cm) had been transported to the lower part (1 cm) in about 2 hrs This part (B 3) contained at this time about the same amount of accumulated NA as part A (Table I) Histochemically, the accumulations above the two ligations exhibited the same appearance at 1 hr after the operation while at 3 hrs the accumulation above the high ligature was larger than above the low ligature Also the accumulations above the low ligature was somewhat larger at 3 hrs than at 1 hr The proximal accumulations above the high and low ligatures thus followed each other for a period of about 2 hrs after the operation These observations may indicate that the rate of convection in the part of nerve between the ligations may be about 1 cm per hr which is the same value as that obtained from calculations performed on the accumulation of NA above a single constriction (Dahlström and Häggendal 1966) Likewise, in rat sciatic nerve the accumulation in the part above the low constriction histochemically followed the course in the part above the high constriction for the first 2—3 hrs and thereafter it stayed unchanged *Since the ligations were placed at a distance of 1 5—2 cm from each other* this observation supports the figure on NA transport of about 5 mm per hr found for the rat in the earlier study

A similar transport between two simultaneous ligations has been observed to occur for AcChE in dog peroneal nerve (Lubinska *et al* 1964) Also here was at 18 hrs observed an increase in AcChE-activity at both sides if the two ligations, the one above each constriction being larger than the increase below each ligature However, the retrograde accumulations of AcChE was found to be considerably

larger compared to the proximal ones than has been found for NA in rat and cat sciatic nerves (Dahlstrom and Haggendal 1966, see also Results)

The spectrophotofluorimetric readings of some of the samples, *e g* parts B 1, B 2 and C in the cat, gave values which were only slightly higher than the values of the tissue blanks Therefore, these very low NA values for the mentioned nerve parts may be considered somewhat uncertain On the other hand, as seen from Table I, the sum of the NA values of the B-parts were about the same as the NA content of normal cat nerves of the same length as the B part In an earlier study, where the nerve parts between two ligations of both rat and cat were determined undivided, as a whole piece at 12 and 18 hrs after the operation, the NA values of these pieces never exceeded the normal NA levels (Dahlstrom and Häggendal 1966) These facts support the NA figures obtained in this study Thus, it may be concluded that between 0—18 hrs there occurs no increase of NA in the sciatic nerve of rat and cat after the ligation procedure

A reason why some NA remained in parts B 1 and B 2 after the ligation may be that an uninterrupted supply of axoplasmic material from above is needed for the normal efficiency of the 'peristaltic waves" As mentioned above (see Results) a few very weakly fluorescent fibres were also observed in the middle part (corresponding to B 2, Fig 1) in the nerves at 3 hrs Another factor of importance may be that the nerves are supplied with blood vessels with adrenergic terminals Since calculations on the rate of transport have been performed using the NA-value for a normal 1 cm piece of nerve, this would mean that the rate of transport may be even somewhat larger than 5 and 10 mm per hr, respectively

The results of the present investigation support the view that the transport mechanism for the amine granules is in main independent of the cell body Furthermore, the obtained results give support to the data on the rate of transport of NA granules in rat and cat sciatic nerves obtained earlier (Dahlstrom and Haggendal 1966)

To Dr J Häggendal Department of Pharmacology Goteborg I would like to express my sincere gratitude for valuable discussions and economical support

The skilful technical assistance of Mrs Mirta Baidins and Miss Ulla Pia Hernio is greatfully acknowledged

This study was supported by a grant from the Swedish State Medical Research Council (B67 14X 166-03 and by the Swedish Government Funds for Supporting Medical Research

References

CARLSSON A Drugs which block the storage of 5 hydroxytryptamine and related amines In *Handbuch der Exp Pharmacol* Ed V Erspamer Springer Verlag Berlin Gottingen Heidelberg 1965

CORRODI H N A HILLARP and G JONSSON Flurorescence methods for the histochemical demonstration of monoamines 3 Sodium borohydride reduction of the fluorescent compounds as a specificity test *J Histochem Cytochem* 1964 *12* 582—586

D [illegible]

D [illegible]

DAHLSTRÖM A and K FUXE A method for the demonstration of adrenergic nerve fibres in peripheral nerves *Z Zellforsch* 1964 b *62* 602—607

DAHLSTRÖM A and K FUXE, Evidence for the existence of monoamine containing neurons in the central nervous system I Demonstration of monoamines in the cell bodies of brain stem neurons *Acta physiol scand* 1964 c *62* Suppl 232 1—55

DAHLSTRÖM, A and J HÄGGENDAL, Studies on the transport and life-span of amine storage granules in a peripheral adrenergic neuron system *Acta physiol scand* 1966 *67* 278—288

ERNYEI S and M R YOUNG Pulsatile and myelin forming activities of Schwann cells *in vitro* *J Physiol* 1966 *183* 469—480

EULER, U S v and N Å HILLARP Evidence for the presence of noradrenaline in submicroscopical structures of adrenergic axons *Nature* (Lond) 1956 *177* 44—45

HAMBERGER, B, T MALMFORS and CH SACHS Standardization of paraformaldehyde and of certain procedures for the histochemical demonstration of catecholamines *J Histochem Cytochem* 1965 *13* 147

HAYASHI T Role of the cortical gel layer in cytoplasmic streaming In *Primitive motile systems in cell biology* Eds R D Allen and M Kamiya Academic Press New York and London 1964 pp 19—29

HILLARP N Å Some problems concerning the storage of catecholamines in the adrenal medulla *Ciba Foundation Symposium on Adrenergic Mechanisms* Eds G E W Wostenholme and M O Connor London J and A Churchill Ltd 1960 pp 481—486

HILLARP, N Å K FUXE and A DAHLSTRÖM, Central monoamine neurons In *Mechanisms of Release of Biogenic Amines* Eds U S v Euler, S Rosell and B Uvnas Pergamon Press Oxford 1966 31—56

HYDÉN H, The Neuron In *The Cell IV* Eds J Brachet and A Mirsky Academic Press New York—London 1960 pp 215—323

HÄGGENDAL, J An improved method for fluorimetric determination of small amounts of adrenaline and noradrenaline in plasma and tissue *Acta physiol scand* 1963 *59* 242—254

KOENIG, E Synthetic mechanisms in the axon II RNA in myelin free axons of the cat *J Neurochem* 1965 *12* 357—361

LUBINSKA L, S NIEMIERKO B ODERFELD L SZWARC and J ZELENA Bidirectional movements of axoplasm in peripheral nerve fibres *Acta Biol exp* (Wars) 1963 *23* 239—247

LUBINSKA, L S NIEMIERKO B ODERFELD and L SZWARC Behaviour of acetylcholinesterase in isolated nerve segments *J Neurochem* 1964 *11* 132 138

MALMFORS T Studies on adrenergic nerves The use of rat and mouse iris for direct observations on their physiology and pharmacology at cellular and subcellular levels *Acta physiol scand* 1965 *64* Suppl 248 1—93

NORBERG K A and B HAMBERGER The sympathetic adrenergic neurons Some characteristics revealed by histochemical studies on the intra neuronal distribution of the transmitter *Acta physiol scand* 1964 *62* Suppl 238 1—42

OCHS S Beading phenomena of mammalian myelinated nerve fibres *Science* 1963 *139* 599—600

THIEME G Small tissue dryers with high capacity for rapid freeze-drying *J Histochem Cytochem* 1965 *13* 386—389

WEISS P The concept of perpetual neuronal growth and proximo-distal substance convection In *Regional Neurochemistry* Eds S S Kety and J Elkes Pergamon Press New York London 1961 pp 220—242

WEISS P A C TAYLOR and P A PILLAI The nerve fibres as a system in continuous flow microcinematographic and electron microscopic demonstrations *Science* 1962 *136* 330

Acta physiol. scand. 1967. 69. 167—179

From the Department of Histology, Karolinska Institutet, Stockholm, Sweden

The Effect of Reserpine and Tetrabenazine on the Accumulation of Noradrenaline in the Rat Sciatic Nerve after Ligation

By

ANNICA DAHLSTRÖM

Received 19 September 1966

Abstract

DAHLSTRÖM, A., *The effect of reserpine and tetrabenazine on the accumulation of noradrenaline in the rat sciatic nerve after ligation.* Acta physiol. scand. 1967. 69. 167—179.

The accumulation of noradrenaline (NA
intervals after the adm
metho
abolis
malan
above ... hrs after ad
reserpine. The first signs of the reappearance
it could be seen above a ligation performed
in the sciatic nerve was observed in animals t
the adrenergic nerves in the sciatic nerve t
these
restit
NA al
doubl
hrs. T
in the ... animals

In accordance with the axoplasm flow theory of Weiss (1961) there is now strong evidence for a proximo-distal transport (Dahlström and Häggendal 1966 a) of amine storage granules (Euler and Hillarp 1956) in the adrenergic neuron. If a ligation is performed on a peripheral nerve, carrying adrenergic fibres, there rapidly occurr large accumulations of noradrenaline (NA) in bulgy, distorted axons above the ligature (Dahlström and Fuxe 1964 a, Dahlström 1965). There is strong evidence for the view that the accumulated NA is bound within amine granules (see Discussion, Dahlström 1965). Since the granules are complex protein structures (Hillarp 1960 a, b) they are in all probability synthesized in the cell body where high amounts of RNA is found (Hydén 1960). Support to this view has been offered by the finding that after a total depletion of the catecholamines (CA) by reserpine the first signs

of a recovery of t a NA in central adrenergic neurons can be seen just around the nucleus, in the Golgi region (Dahlstrom, Fuxe and Hillarp 1965) Since reserpine is known to be a long lasting blocker of the amine storage mechanism of the granules (Carlsson, Hillarp and Waldeck 1963, Carlsson 1965) this view seems reasonable Tetrabenazine, on the other hand, which is known to be a short acting blocker of the storage mechanism (*cf* Pletscher, Brossi and Gey 1962) caused quite another picture of NA recovery The reappearance of the transmitter could be observed to occur simultaneously in all parts of the neuron (Dahlstrom, Fuxe and Hillarp 1965) indicating a simultaneous recovery of the storage ability of the granules in the whole neuron

Since the transport down the axon of amine granules is apparently very rapid (Dahlstrom and Haggendal 1966 a) it was thought of interest to study the influence of a long- and a short lasting granule blocking agent (reserpine and tetrabenazine, respectively) on the accumulation of NA above a ligation performed at *different* times before and/or after drug administration, systemic and local The method used has been the histochemical fluorescence method of Hillarp and coworkers (for references see Hillarp, Fuxe and Dahlstrom 1965 Corrodi and Jonsson 1966)

Material and methods

Male albino rats of the Sprague Dawley strain (180—220 g) were used Reserpine (Serpasil®,

was never left *in situ* and great care was always taken when the ligation was performed to cause the least possible damage of the blood vessels in the nerves The drug treated animals ere kept at a temperature of 28—30 C, except some which were kept at normal room temperature (19—21 C)

In one group of animals reserpine was topically applied at different times before ligat n (Table IV) on the ganglia in the abdominal sympathetic chain (3 pairs) from which about 90 per cent of the adrenergic fibres in ... The animals were operated un... including the left kidney ... in place by a moist piece ... of blunt forceps Small cotton balls were damped in Serpasil® solution (ampoules conta ing 2 5 mg/ml), squeezed gently before application to remove excess Serpasil and gently put on the ganglia The balls were removed after 4— min and the peritoneal area around the g nglia was carefully washed with an isotonic glucose solution Control animals were treated the a e way but with the solution medium (Losungsmittel Reserpins CIBA) on the sympathet gangl a The peritoneal cavity was closed with silk sutures and the skin with agraffes Most animal howed a slight general reaction to the treatment immediately after the operation but 3—4 hrs later the appearance and behaviour were normal A few animals showing slight reserpine syndromes at 3 9 hrs after the treatment were excluded The animals were killed by a blow on the head 1 h after a high and a low ligation of the left and right sciatic nerve respectively (see Fig 9A The nerves were rapidly dissected out in pieces of about 1 cm length gently stretched out on a piece of paper and im ediately frozen in liquid propane cooled by liquid nitrogen The ganglia of all animals treated w th reserpine ... After freeze-drying ... gas of ... paraffine ... ted f r ... 4 Nor

TABLE I The depleting effect of reserpine given intraperitoneally (10 mg/kg) on the accumulation of NA above single and double (Fig 9 B) ligations of the rat sciatic nerve Normal fluorescence intensity + + + Number of animals within brackets

	Interval ligation — reserpine treatment (hrs)	Interval reserpine treatment — sacrifice (hrs)	Fluorescence intensity	
Single lig	6	6 (4)	0	
	12	12 (3)	0 → (+)	
	12	6 (5)	0 → (+)	
	0	12 (6)	0 → (+)	
	[1]3	6 (3)	++ → +++	
	3	6 (3)	0	
			upper lig	lower lig
Double lig	4 (3)	4	0	0
	6 (4)	6	0	0
	[2]8 (2)	—	+++	++

[1] The animals were given nialamide (250 mg/kg i p) at the same time as the ligation was performed

[2] The animals were double ligated only at 8 hrs before sacrifice to serve as controls for fluorescence intensity

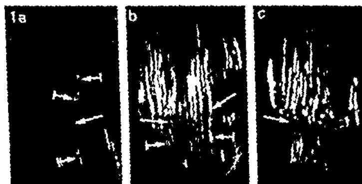


Fig 1 The effect of reserpine and nialamide on the accumulation of NA above ligations of the sciatic nerve of rat a The nerve was ligated 12 hrs before an i p injection of reserpine (10 mg kg) given 6 hrs before the sacrifice Above the lesion (→) only a few very weakly fluorescent nerve fibres can be seen (→ Below the lesion accumulations of material with an unspecific brownish green yellow fluoresence are observed → b The animal was pretreated with the MAO inhibitor nialamide 250 mg kg at the time of ligation Reserpine 10 mg kg was given i p 3 hrs after the pretreatment and the animal was killed 6 hrs later The treatment with nialamide had prevented the emptying effect of reserpine and strongly green fluorescent material can be observed in distended nerve fibres above the lesion → Below the lesion retrograde accumulations with specific green fluorescence of weak to medium intensity are seen (→) c The accumulation of NA in a normal rat with 9 hrs ligation of the sciatic nerve The ligation is indicated (→) As seen from Fig b and c the accumulations of NA in the 2 rats have about the same appearance

Fluorescence microphotographs 25

TABLE II The effect of an intraperitoneal injection of reserpine (10 mg/kg) on the accumulation of NA in the sciatic nerve of rat The ligation of the nerve was performed 1 hr before sacrifice, high at the left side and about 1 5—2 cm lower at the right side Number of animals within brackets

Interval reserpine sacrifice	Fluorescence intensity	
	High lig	Low lig
0 (7)	+ + +	+ + +
3 (6)	(+)	(+)
6 (6)	0	0
9 (6)	0	0
12 (8)	0	0
15 (10)	(+)	0
18 (10)	(+) → +	(+)
21 (10)	+ → +(+)	(+) → +(+)
24 (8)	+(+) → ++	+(+) → ++
30 (6)	++ → +++	++ → +++
36 (4)	+++	+++

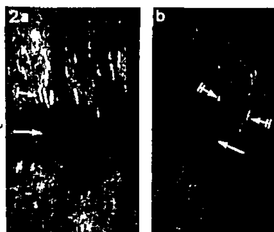


Fig 2 The accumulation of NA in the sciatic nerve of a rat given reserpine 18 hrs before sacrifice The ligations of the nerves were performed 1 hr before death high at the left side (a) and about 2 cm lower at the right side (b) The amount of accumulated fluorescent material is larger above the high lesion in a) (⟼) than above the low lesion in b) (⇸) The site of the lesions is indicated (—•) No retrograde accumulations can be observed
Fluorescence microphotographs × 68

Results

Reserpine *Depletion* Reserpine given to rats in which NA had already accumulated caused the fluorescence to disappear almost entirely after 6—12 hrs (Table I, Fig 1 a) Also the small retrograde accumulation (see Dahlström 1965) lost the specific green fluorescence, only signs of a brownish yellow-green fluorescence being observed under a single ligature In rats with double ligations on the same nerve 1—2 cm apart, reserpine had emptied the accumulation above and below both ligatures 4—6 hrs after the injection (see Table I) Administration of the MAO-inhibitor nialamide (250 mg/kg *i p*) given at the same time as the ligations were performed prevented the disappearance of NA otherwise occurring in animals after reserpine treatment (Table I, Fig 1 a—c)



Fig 3 The effect of reserpine (2 mg kg) on the restituted fluorescence above a ligation performed 24 hrs after pretreatment with a first dose of reserpine (10 mg/kg) a) The accumu [illegible] (⟶) b) The effect of a second dose of reserpine (2 mg/kg) given to an animal treated as decribed in a) The second reserpine injection was given 6 hrs after the ligation and the rat was sacrificed 12 hrs later No fluorescent material can be observed above the ligation (⟶) Below the ligation material with unspecific yellow brown fluorescence is seen (↦)
Fluorescence microphotographs × 25

TABLE III The effect of reserpine given intraperitoneally on the accumulation of NA above single and double ligations of the rat sciatic nerve Normal fluorescence intensity + + + Number of animals within brackets

Interval reserpine inj — ligation (hrs)	*Interval ligation — death (hrs)*	*Fluorescence intensity*	
Single lig			
24	[1]18 (4)	0	
24	6 (3)	+ + → + + +	
24	12 (3)	+ + → + + +	
	[2]24 (3)	+ → + +	
Double lig		Upper lig	lower lig
12	6 (4)	(+) → +	0
12	9 (4)	+ → + (+)	0
15	6 (4)	+ → + (+)	0 → (+)
	[3] 6 (3)	+ + +	+ +

[1] The animals were given a second dose of reserpine (2 mg/kg) 12 hrs before death
[2] The animals were given reserpine (10 mg/kg) at the same time as the ligation was performed
[3] The animals were double ligated only to serve as controls for fluorescence intensity

Recovery Ligations (the left nerve high and the right one 1 5—2 cm below the left ligature Fig 9 A) were performed at different intervals after reserpine (10 mg/kg) and the animals were killed 1 hr later (Table II) The fluorescence above the ligations was seen to have disappeared within 3—6 hrs Recovery of fluorescence above the constrictions could be observed 16—18 hrs after the administration of the drug, and was usually observed in the high ligated nerve before it appeared in the low ligated nerve (Fig 2 a, b) At 18—21 hrs after the injection of reserpine the fluorescence intensity was often stronger above the high ligature than above the low one, but from 24 hrs on no difference could be observed (Table II) No retrograde accumulation could be seen in these early phases of recovery The increase in fluorescence

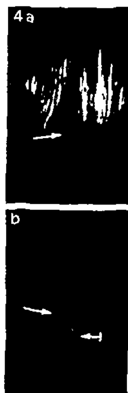


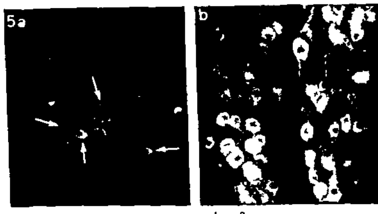
Fig 4 The reappearance of fluorescence (NA) in a double ligated sciatic nerve of rat after reserpine treatment (10 mg/kg i p) The rat was given reserpine 12 hrs before the performance of the two simultaneous ligations and killed 9 hrs after the ligation Only above the upper ligature in a) accumulations of fluorescent material has reappeared Above the lower ligature in b) the downtransport of newly formed granules was inhibited by the upper ligature and no fluorescence can be observed Below the lower ligation fibres with unspecific fluorescence are indicated (↦) The site of the ligations is indicated (⟶) Fluorescence microphotographs × 25

intensities continued gradually, and control levels appeared to be reached 30—36 hrs after reserpine The animals treated in exactly the same way as above but kept at 20 C showed a slower recovery than those kept at 28—30° C The first recovery of fluorescence in these animals was observed at 18—21 hrs

Animals given reserpine (10 mg/kg) 24 hrs before ligation and killed 6—12 hrs later showed a moderate to strong fluorescence intensity in rather large bulgy fibres above the ligature, and a weak fluorescence intensity in the retrograde accumulation below the ligature (Fig 3 a, Table III) If a second dose of reserpine (2 mg kg) was given 6 hrs after ligation no fluorescence was observed 12 hrs later (Fig 3 b

In the double ligated rats, operated 12 and 15 hrs after reserpine treatment and killed 6 and 9 hrs later, recovery of fluorescent material was observed only above the upper ligature (Fig 4 a) Above the lower ligature, no increase in the very weak to absent fluorescence occurred during the 6—9 hrs after ligation (Fig 4 b Table III) In animals given reserpine at the time of the ligation, 24 hrs before sacrifice, the fluorescence was of medium intensity, the fluorescence-containing fibres having bulgy appearance for a considerable length

Local application Experiments with local applications as performed in this study did not lead to a successful depletion of all the ganglion cells in more than about 60 per cent of the animals The remaining 40 per cent were excluded Further 10 per cent were excluded because they showed slight systemic effects of reserpine



due to fluorescence
Fluorescence m crophotographs × 155

TABLE IV The effect of local appl cat on of reserp ne at the ganglia on the accumulation of NA n the sc at c nerve of rat The l gation of the nerve was performed 1 hr before death h gh at the left s de and 1 5 2 cm lower at the right s de Only an mals showing a good effect of reserp ne n the gangl a but without notable system c effects were included in the table Number of an mals with n brackets

In erval reserp ne appl cat on death	Fluorescence intens ty H gh l g	Low l g
0 (5	+++	+++
3 6	+ → ++	+ → ++
6 6	0 → (+)	(+)
9 6	0	0
12 (8	(+)	0
15 7	(+) → +	(+)
18 7	+ → (+)	+
21 6	+ → ++	+ → ++
24 (	++ → +++	++ → +++

The gangl a of these rats were treated w th the sol ent of reserp ne 9 hrs before sacrifice

Gangl a The ganglia of all rats were stud ed A total depletion could be observed from 3 to 6 hrs wh le a small recovery of fluorescence was seen to appear at 9—12 hrs in many of the cells The first fluorescence was often observed around the nucleus of the ganglion cells and later it gradually filled the perikaryon Animals with ganglia contain ng many cells with a weak green fluorescence all over the cell body at 12—21

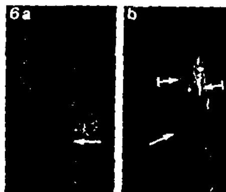


Fig 6 Sciatic nerves, ligated 1 hr, of a rat locally treated with reserpine on the ganglia 6 hrs before [illegible]

hrs after the local application of reserpine were discarded, since total depletion possibly never had occurred Such animals showed largely unaffected accumulations of fluorescent material above the ligations Also after *i p* injections of reserpine ring structures of fluorescent material could often be seen around the nucleus of many cells (Fig 5 a), as observed in central *CA* neurons (Dahlstrom, Fuxe and Hillarp 1965) after reserpine treatment The ganglia of the control animals, treated with the solvent showed a normal appearance (Fig 5 b)

Sciatic nerves The disappearance of the fluorescence above the ligations occurred somewhat later than in the *i p* injected animals (Table IV) At 3 hrs a decrease to medium fluorescence intensity was seen at both sides, while at 6 hrs the intensity was very weak, especially above the high ligature In some animals at 6 hrs no fluorescence was observed above the high ligature while weak accumulations could be seen above the low ligature (Fig 6 a, b) *The recovery followed the pattern of* that observed in the systemically treated animals but occurred generally about 3 hrs earlier and appeared to be complete after 24 hrs (see Table IV) In sciatic nerves from these topically treated animals there was often (also at 6—15 hrs observed a few fibres with NA accumulations of normal appearance, usually situated in the periphery of the nerves These fibres in all probability represent a part of the 10 per cent of NA fibres which do not have their cell bodies in these ganglia Dahlstrom 1966, unpubl In the nerve, blood vessels were often observed with adrenergic *nerve terminals of a normal appearance, showing that little or no obvious systemic* effects had developed after the local treatment

Tetrabenazine *Depletion* Tetrabenazine given to rats ligated at different times before drug-administration, caused within 4 hrs a rapid and pronounced decrease in the fluorescence intensities of the accumulations above and below both ligatures (Fig 7 a, b, Table V

Recovery Recovery of the fluorescence intensities occurred at 10—15 hrs after the injection of tetrabenazine (Table V), both above the high and above the low *ligature in double ligated animals* (Fig 8 a, b) Also the retrograde accumulations below both constrictions had regained their specific, weak green fluorescence

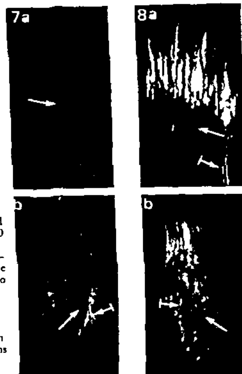


Fig 7 Double ligated sciatic nerve of a rat sacrificed 4 hrs after the injection of tetrabenazine (100 mg/kg) The animal was ligated 4 hrs before the drug administration a) Upper ligation (⟶) The accumulation of NA developed during the [illegible]

fluorescence can be observed (↦) Fluorescence microphotographs × 25

Fig 8 Double ligated sciatic nerve of a rat ligated 3 hrs after tetrabenazine administration (100 mg kg i p) and killed 12 hs after the ligation a) Upper ligation (⟶) Strongly green fluorescent material accumulated within bulgy nerve fibres can be observed above the ligation Also [illegible]

thin, somewhat bulgy fibres above the ligation Also below the lesion retrograde accumulations *of specific green fluorescence can be seen* (↦) Fluorescence microphotographs × 25

TABLE 1 The effect of tetrabenazine (100 mg kg i p) on the accumulation of NA in single and double ligated rat sciatic nerves Number of animals within brackets Normal fluorescence intensity + + +

Depletion	Interval ligation — tetrabenazine injection (hrs)	Interval tetrabenazine injection — death (hrs)	Fluorescence intensity	
Single lig	6	4 (3)	0 → (+)	
			upper lig	lower lig
Double lig	3	4 (4)	0	0
Recovery	**Interval tetrabenazine injection — ligation (hrs)**	**Interval ligation — death (hrs)**	**Fluorescence intensity upper lig**	**lower lig**
Double lig	3	12 (4)	+ + +	+ (+) → + +
	4	6 (4	+ → + +	(+) → +
		[1]6 (2)	+ + +	− +

[1] The animals were double ligated only to serve as controls for fluorescence intensity

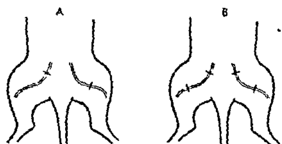


Fig 9 Schematic illustration of the different types of ligations performed at the sciatic nerves of the rats The figures are made from a dorsal view A Single ligations performed at different level of the sciatic nerve The left nerve is ligated about 1 5—2 cm higher than the right nerve B Double ligations performed at the same nerve Both sciatic nerves are ligated at two places each the lower ligation situated about 1 5—2 cm below the upper ligation

Discussion

The histochemical fluorescence method of Hillarp and co-workers (for description and references see Hillarp Dahlström and Fuxe 1965 Corrodi and Jonsson 1966) is highly specific for the demonstration of catecholamines and 5 hydroxytryptamine (see Corrodi Hillarp and Jonsson 1964 Corrodi and Jonsson 1965) It is concluded that the green fluorescent material accumulated above the ligature is NA (see Dahlstrom 1965 Dahlstrom and Haggendal 1966) Since the method also has a great sensitivity very small amounts of NA can be observed in the microscope see *e g* Norberg and Hamberger 1964)

The mode of action of reserpine has been shown to be a blockage of the amine storage mechanism in the granules and this may well apply also for tetrabenazine (*cf* Pletscher Brossi and Gey 1962 Carlsson Hillarp and Waldeck 1963 Lundborg 1963 The effect of reserpine is long lasting (*cf* Dahlstrom and Fuxe 1964 a Norberg and Hamberger 1964 Carlsson 1965) while that of tetrabenazine is short lasting (Pletscher Brossi and Gey 1962) Evidence has earlier been put forth for the view that the complex amine storage granules are formed in the cell body around the cell nucleus (Dahlstrom Fuxe and Hillarp 1965) Support for the existence of a rapid transport of these granules has also been obtained Dahlstrom and Fuxe 1964 b Dahlström 1965 Calculations of the rate of this transport have been made Dahlström and Haggendal 1966) and found to be about 5 mm per hr for the rat It has also been shown that the transport mechanism is mainly independent of the cell body since a proximo-distal convection occurs also in the nerve part between two simultaneously performed ligations Dahlstrom 1966) Fig 9 B) The observations in this study all fit in with, and strongly support the above mentioned findings on the transport of amine granules The mode of action of the drugs used together with the view of the site of synthesis and transport of storage granules seems to afford a reasonable explanation to the obtained results mentioned below

1) Both reserpine and tetrabenazine caused a strong decrease of the fluorescence intensity in the accumulation above a ligature The amines stored in the granules had in all probability leaked out into the axoplasm where they were rapidly deaminated

by MAO The depleting action of reserpine was at least to a large part prevented when MAO inhibition had been performed prior to the reserpine injection These observations support the view that the NA of the accumulations is granule bound In animals with two simultaneous ligations of the same nerve the two drugs abolished the fluorescence almost entirely also in the accumulation above the lower ligature, indicating that also here the amines existed granule bound

2) The first signs of recovery from reserpine treatment appeared as fluorescence in the nerve above the high ligation before it appeared above the low ligation, provided that the level difference between the ligations was large enough (more than 1 5 cm) The only reasonable explanation for this seems to be that newly formed granules with an intact storage mechanism had been transported down the axon from the cell body and had consequently at first been arrested by the highest ligature In animals with double ligations the accumulation above the low ligature did not regain its fluorescence within the 12 hrs after ligation (up to 24 hrs after reserpine) studied while the accumulation above the upper ligature showed weak to medium fluorescence intensities The reason for this is in all probability that the high ligature had prevented the further down transport of newly formed granules, necessary for a reappearance of the NA

3) A second dose of reserpine to animals showing recovery of fluorescence in the ligated nerve was able to completely deplete the recovered fluorescence This indicates that also under the early recovery phase the amines are located in storage granules

4) In animals the ganglia of which had been depleted by local application the recovery of fluorescence above a 1 hr ligation followed the same pattern as in the systemically treated rats The disappearance of the fluorescence above the constrictions however in all rats occurred some hours later than in the *i p* injected rats and in some animals at 6 hrs it was clearly seen that the fluorescence above the high ligature was absent while there still was a visible specific fluorescence above the low ligature Also the nerve terminals of the blood vessels and a few fibres with a peripheral location showed a normal appearance in the microscope This indicates that the disappearance of NA in the adrenergic axons was due to the circumstance that the unblocked NA containing granules in the axon had been transported further down the axon and empty reserpine blocked granules had instead been transported from the cell bodies to the site of ligation

5) The short acting blocker tetrabenazine caused a total abolishment of the fluorescence above and below both constrictions in double ligated rats But in spite of the high ligature the accumulation above the low ligature showed a histochemical recovery at 10 and 15 hrs after the injection of the substance and at the same time as the accumulation above the high ligature This may indicate that the amine granules in the separated nerve part were fairly normal in respect of synthesis and storage of NA

In general tetrabenazine effects NA levels in peripheral tissues of *i a* guinea pig and rabbit less than in the brain (Pletscher Brossi and Gey 1962) However biochemical

data on the action of this drug on NA levels in rat tissues at the dosage and time intervals employed do not seem to be available Certain histochemical observations (Malmfors, pers comm), however, indicate that tetrabenazine has an action in rat peripheral tissues

Taken together, the results obtained in this study give further strong support for the view that the amine storage granules are formed in the cell body and transported distally through the axons at a high rate Furthermore, they indicate that within 1—3 days after reserpine treatment, newly formed, intact granules have already started to reach the nerve terminals The recovery of NA in the terminals after reserpine treatment may subsequently be related to the gradual exchange of old, reserpine blocked granules by newly formed ones (Dahlström and Haggendal 1966 b)

I wish to express my gratitude to Mrs Mirta Baidins and Miss Ingalill Nordgren for skilful technical assistance

For generous supplies of drugs I am indebted to Swedish CIBA Stockholm (reserpine), Swedish Pfizer Stockholm (nialamide) and Roche Produkter AB Stockholm (tetrabenazine)

The study was supported by a grant from the Swedish Medical Council

References

CARLSSON A Drugs which block the storage of 5 hydroxytryptamine and related amines In *Handbuch der Exp Pharmacol* Ed V Erspamer Springer Verlag Berlin Gottingen Heidelberg 1965

CARLSSON A N Å HILLARP and B WALDECK Analysis of the Mg ATP dependent storage mechanism in the amine granules of the adrenal medulla *Acta Physiol Scand* 1963 *59* Suppl 215 1—38

CORRODI H and G JONSSON Fluoreszensmethoden zur histochemischen Sichtbarmachung von Monoaminen 5 Identifizierung des fluoreszierenden Produktes aus Modellversuchen mit 5 Methoxytryptamin und Formaldehyd *Acta Histochem* 1965 *22* 247—258

CORRODI H and G JONSSON The fluorescence method for the histochemical demonstration of monoamines Methodology *J Histochem Cytochem* (in press)

CORRODI H N Å HILLARP and G JONSSON Fluorescence methods for the histochemical demonstration of monoamines 3 Sodium borohydride reduction of the fluorescent compounts as a specificity test *J Histochem Cytochem* 1964 *12* 582—586

DAHLSTROM A Observations on the accumulation of noradrenaline in the proximal and distal parts of peripheral adrenergic nerves after compression *J Anat* (Lond) 1965 *99* 677—689

DAHLSTROM A The transport of noradrenaline between two simultaneously performed ligations of the sciatic nerve of rat and cat *Acta physiol scand* 1967 *69* 158 166

DAHLSTROM A and K FUXE Existence of monoamine containing neurons in the central nervous system I Demonstration of monoamines in the cell bodies of brain stem neurons *Acta physiol scand* 1964 a *62* Suppl 232 1 55

DAHLSTRÖM A and K FUXE A method for the demonstration of adrenergic nerve fibres in peripheral nerves *Z Zellforsch* 1964 b *62* 602—607

DAHLSTRÖM A K FUXE and N Å HILLARP Site of action of reserpine *Acta pharmacol* (Kbh) 1965 *22* 277—292

[illegible] granules [illegible] with [illegible] -752 [illegible] scop [illegible]

HAMBERGER, B , T MALMFORS and CH SACHS Standardization of paraformaldehyde and of certain procedures for the histochemical demonstration of catecholamines *J Histochem Cytochem* 1965 *13* 147

HILLARP, N Å, Some problems concerning the storage of catecholamines in the adrenal medulla *Ciba Foundation Symposium on Adrenergic Mechanisms* Eds G E W Wolstenholme and M O Connor J and A Churchill Ltd London 1960 a p 481—486

HILLARP N Å Catecholamines mechanisms of storage and release *Acta endocrin* First International Congress of Endocrinology Copenhagen Lect. 9 1960 b pp 181—185
HILLARP N Å, DAHLSTROM A and K. FUXE, Central monoamine neurons In *Mechanisms on Release of Biogenic Amines* Eds U S v Euler S Rosell and B Uvnas Pergamon Press 1966 pp 31—57
HYDEN H The neuron In *The Cell* IV, Eds J Brachet and A Mirsky Academic Press New York London 1960 pp 215—323
LUNDBORG P Storage function and amine levels of the adrenal medullary granules at various

stics
Acta

mine

hem

Acta physiol. scand. 1967, 69, 180—186

From the Institute of Occupational Health, Helsinki, Finland

Constancy of d(+)Xylose Space in Man during Brief Exercise

By

J. HASAN, M. J. KARVONEN, P. PIIRONEN and R. RUOSTEENOJA

Received 28 September 1966

Abstract

HASAN, J., M. J. KARVONEN, P. PIIRONEN and R. RUOSTEENOJA. *Constancy of d(+)xylose space in man during brief exercise.* Acta physiol. scand. 1967. 69. 180—186.
Measuring the concentration of xylose in blood at intervals between the 15th and 65th minute [illegible] any increase in the apparent [illegible] have exceeded approximately [illegible] of the subjects' average body [illegible] ditions failed to confirm the [illegible] contractions of anesthetized animals that the permeability of the cell membrane to sugars is increased to a physiologically important extent during muscular exercise, the alteration allegedly resembling closely that brought about by insulin. We conclude that the discrepancy between the present findings and those reported previously may be due to an interspecies difference in mechanisms of regulation or, rather, it reflects the fact that previous experiments involved far from physiological conditions, likely to induce e.g. fluid shifts and consequent distortions of interpretation.

After Levine and his co-workers had reported on the increased volume of distribution of several non-utilizable sugars in the eviscerated rat, brought about by an injection of insulin (Levine *et al.* 1950, Goldstein *et al.* 1953 a), observations were extended to show a similar effect of electrically induced muscular contractions in anaesthetized, wholly or partially eviscerated animals (Goldstein *et al.* 1953 b, Levine and Goldstein 1955, Helmreich and Cori 1956, 1957, Sacks and Smith 1958). This activity was reported to increase the volumes of distribution of several sugars, including pentoses, structurally more or less related to d(+)glucose. Among these d(—)xylose is of special interest because of its slow utilization in the preparations used, combined with its ready responsiveness to the actions of insulin and muscular contractions (Kipnis and Cori 1957). It has been claimed that the mechanisms involved affect only the distribution of sugars, which have a steric con-

figuration on their first three carbon atoms identical to that of d(+)glucose (Goldstein *et al* 1953 a and b, Sacks and Smith 1958), but this has been refuted (Helmreich and Cori 1957, Drury and Wick 1955, Park *et al* 1956) The implication seems to be that the mechanisms of action of both insulin and muscular activity might be identical, despite some evidence to the contrary (Sacks and Smith 1958) It has not been possible to establish unequivocally whether the effect observed with electrically induced muscular activity is due to local changes in the contracting cells (Sacks and Smith 1958, Morgan *et al* 1965) or mediated, perhaps, through some humoral agent (Goldstein *et al* 1953 b, Helmreich and Cori 1957, Goldstein 1960), the confusion may be due also to the variations in animal species and preparations studied (Segal *et al* 1957, Sacks and Smith 1958) Glucose uptake into frog striated muscle has been reported unresponsive to both insulin and induced contractions *in vitro* (R Candela *et al* 1962)

Man is naturally of special interest even in this respect, and for experimental purposes he may easily be subjected to an accurately adjusted amount of muscular activity Segal *et al* (1957) have demonstrated that in man insulin decreases the plasma concentrations of infused, slowly metabolized sugars, such as d(+)xylose, presumably by increasing its volume of distribution into the intracellular space, corresponding to the effect found in animals *in vivo* (Goldstein *et al* 1953 a, Helmreich and Cori 1957, Sacks and Smith 1958) and *in vitro* (Park *et al* 1956, Kipnis and Cori 1957, Eichhorn and Hechter 1961, Carlin and Hechter 1961, Morgan *et al* 1961) If the postulated effect of muscular activity on the permeability of cells to sugars were true in physiological conditions, it could be expected to be demonstrable in man with d(+)xylose We have therefore made a series of experiments in order to study whether previous observations with artificially stimulated muscles in anaesthetized animals or animal preparations could be confirmed with voluntary muscular exercise in man

Material and methods

The subjects were three healthy males of sedentary occupations aged 26 28 and 29 and two well trained students of physical education aged 19 and 20 The experiments were performed at noon with the subjects refraining from eating after 6 a m A large-bore injection needle was placed into a forearm vein of the subject reclining comfortably on a bed in a well ventilated [illegible]

3 min In pr[illegible]
xylose in blo[illegible]
xylose level [illegible]
— was ther[illegible]
10 min inte[illegible]
stoppered b[illegible]

Thirty minutes after the end of the injection the subject mounted a bicycle ergometer A blood sample was drawn and the subject immediately started pedalling at a load of 18 kpm sec at a fixed speed He stopped after 10 min and a blood sample was again drawn with the subject still mounted on the ergometer He then laid down again on the bed and three additional blood samples were collected during the following ?5 min

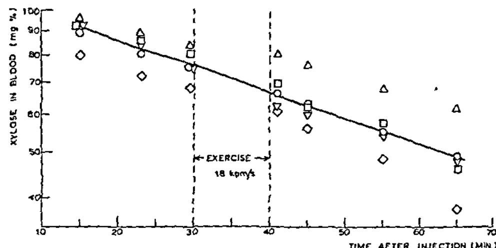


Fig 1 The time course of the concentration of xylose in blood (logarithmic scale) of five healthy men from 15 to 65 min after the i v injection of 12 5 g of d(+)xylose Between the 30th and 40th min after the injection the subjects pedalled on a bicycle ergometer against a load corresponding to 18 kpm sec Individual values for each subject are denoted by symbols and a continuous line has been drawn through the mean values

No systemic ill-effects were observed from the injections or the exercise and the only complaints of the subjects referred to some occasional dull pain at the site of venipuncture signs of slight inflammation could be discerned in the area in two subjects

The blood samples were collected in centrifuge tubes all treated similarly with heparin solution and deproteinized within 30 min of collection by adding equal volumes of stoichiometrically matched 0 3 N $Ba(OH)_2$ and 5 % $ZnSO_4$ (Somogyi 1945) The precipitate formed was filtered off in small paper funnels

A suspension of yeast was prepared by washing ordinary baker s yeast with five times 20 volumes of distilled water spinning the slurry each time down in a centrifuge 1 volume of the washed slurry was finally added to 4 volumes of distilled water 0 5 ml of this suspension was added to 2.5 ml of the deproteinized, filtered blood sample and the suspension was shaken for two hours

from blood

After treatment with yeast the samples of blood deproteinate and the standard solutions were assayed for reducing sugar by the method of Somogyi (1952 using Nelson s (1944) colour reagent and a photoelectric colorimeter Klett Summerson The method error (Fischer 1950 derived from duplicates was 4 mg The experiments reported here were originally performed in the spring of 1957

Results

The results from the five experiments are depicted in Fig 1 The concentration of xylose in the blood of most subjects decreased at an approximately exponential rate between the 15th and 30th min after the injection, with an average half period of about 60 min range 50 to 80 min During their initial course of decline, the blood xylose concentrations of the 5 subjects were within 20 mg 100 ml of each other

Neither visual observation nor statistical computation McNemar 1955 revealed any significant deviation from a straight line in the time course of the logarithm of the

mean concentration of xylose in blood. The divergence between the individual curves, reflected in the range of half periods above, was accompanied by increasing nonlinearity in the later portions of the observed time course of xylose concentration in some subjects. However, no clear acceleration, temporally related to the period of exercise, could be observed in the exponential decline of the subjects' blood xylose concentrations such as would have been expected from a more or less sudden increase in the volume of distribution of xylose in the body (Levine *et al.* 1950).

Discussion

The steady exponential rate of decline of the xylose concentration in blood fifteen minutes after the injection seems to indicate a satisfactory mixing of the injected d(+)xylose in its primary space of distribution (Dominguez, Goldblatt and Pomerene 1937, Dominguez 1950). Extrapolation of the mean curve between 15 and 30 min after the injection to zero yields an initial volume of distribution of about 14.5 l, or approximately 21 per cent of the average body weight of the subjects, which agrees well with previous estimates of the xylose space in man (Wyngaarden, Segal and Foley 1957). This indicates that some xylose is apparently distributed outside the extracellular space, *i.e.* it is slowly permeating into the cells. The exponential decline with time of the blood xylose concentration is predominantly a function of the slow intracellular penetration (Helmreich and Cori 1957), metabolism (Dominguez, Goldblatt and Pommerene 1937, Helmreich and Cori 1957, Wyngaarden, Segal and Foley 1957), and excretion (Dominguez, *et al.* 1937, Wyngaarden *et al.*). The apparently single exponential time course, at least as a first approximation, implies a first order kinetic progress of removal of the pentose from blood. The data do not, however, permit a more detailed and definitive analysis of this question. Previous authors have claimed that xylose is eliminated from dog plasma by a process characterized by two exponentials (Dominguez *et al.* 1937), while more recent experiments have been interpreted as indicating a first order reaction (Holmberg *et al.* 1956); there are however numerous pitfalls in graphical analysis of kinetic data from systems which are incompletely known (Van Liew 1962).

There is no indication of any influence of the exercise on the time course of mean blood xylose concentration. The experiments thus failed to demonstrate a facilitation of intracellular sugar permeation in exercising man. Such an effect has been demonstrated with electrical induction of contractions in striated muscles of eviscerated anesthetized rats (Helmreich and Cori 1957), dogs (Goldstein *et al.* 1953, Goldstein 1960) and cats (Sacks and Smith 1958) as well as in the spontaneously working isolated heart muscle of rats (Morgan *et al.* 1965). Insulin has also been found to increase the volume of distribution of d(+)xylose in all these preparations and in the resting human organism; the latter could therefore have been expected to respond to muscular activity too.

An increase in the volume of distribution of xylose during exercise could be obscured by some other simultaneous alteration if the latter would produce an oppo-

site change of equal magnitude in the blood xylose concentration Of the factors affecting the xylose concentration in blood, metabolic utilization should diminish to be effective in this instance A change in that direction can hardly be postulated with the overall metabolic rate increasing manyfold in muscular exercise Furthermore, the rate of utilization of xylose in the organism is probably limited by transport into the cells (Morgan *et al* 1961)

The urinary output of xylose was not measured in the present experiment, and the possibility remains of a dimished renal excretion of xylose during and immediately after the period of exercise (Ingle, Morley and Stetten 1955), *e g* due to a reduction in renal blood flow (Barclay *et al* 1945, 1947, Chapman *et al* 1948) Xylose is not re-absorbed in the kidneys and its excretion should not be affected by antidiuresis of tubular origin (Keith, Power and Peterson 1934), but a decrease in the glomerular filtration rate has been conclusively established during and immediately after muscular exercise (*e g* Carlsten and Grimby 1966) The extent to which the consequent retardation of xylose excretion may compensate for the effects on plasma xylose concentration of an increase in the volume of distribution of this pentose, may be evaluated as follows The urinary excretion constant of xylose has been found to vary from approximately 0 2 to 0 4 mg/min per unit plasma concentration (Dominguez 1950, Wyngaarden *et al* 1957) We may generously assume that 0 5 represents the maximum in our subjects From Fig 1 it may be seen that during the 12 min time interval between samplings before and after exercise, the mean concentration of xylose in blood was about 70 mg per 100 ml If the slowness of penetration of xylose into erythrocytes is taken into account (Helmreich and Cori 1957) by assuming a mean plasma concentration for the same time interval of 100 mg per 100 ml, at most approximately $0\,5 \times 100 \times 12 =$ 600 mg of xylose would be excreted during the specified period of time Assume this excretion completely inhibited by the exercise, and take the corresponding steady continuity of the exponential decline in the plasma concentration as a result from a concomitant compensatory increase in the apparent volume of distribution of xylose, which is initially 14 5 l on the average Stipulate that in order to indicate a significant alteration in the volume of distribution or kinetics of disposal, a post-exercise blood xylose concentration has to differ from that predicted by the pre-exercise single exponential time course, even when corrected for non-excretion or other known anomalies, by two standard deviations (S D) of the mean from the duplicate determination With a method error (= S D of a single determination) of 4 mg/100 ml, this is about $2 \times 2\,85 = 5\,7$ mg/100 ml 600 mg of xylose distributed evenly into 14 5 l will only increase the concentration by about 4 1 mg% Thus, even if complete cessation of urinary excretion is assumed, the present data do not show that an increase of the xylose space took place during or after the exercise If an intracellular concentration of about 50 mg/100 ml is assumed Helmreich and Cori 1957) it may be stated that any increase in the xylose space of exercising men in the present study cannot have exceeded about 1 7 l, which is less than 12 per cent of its initial volume In the previously reported experiments (Gold

stein *et al* 1953 b, Levine and Goldstein 1955, Helmreich and Cori 1956, 1957, Sacks and Smith 1958) the range of increase in the xylose space of the various preparations was from 50 to 300 per cent Even the weakest stimulus, therefore, brought about a substantially larger alteration than the smallest detectable in the present experiments

It cannot be decided conclusively from the available data whether the failure of human subjects to conform to the other half of this double pattern of xylose responsiveness to insulin and exercise is due to a true interspecies variation, or if it results from differences in the experimental methods It seems clear that the apparently rather violent contractions kept up in the striated muscles of experimental animals by electrical stimulation for prolonged periods of time — *e g* "one or two twitches per second for one hour" (Sacks and Smith 1958) — may well belong to a different category of phenomena from the voluntary muscular exercise performed for ten minutes in our experiments It seems to us that the nature of the former type of activity is such as could be expected to induce alterations in the cellular and/or extracellular tissue spaces (Miller and Darrow 1941, Cullumbine and Koch 1949, Welt *et al* 1950, Jacobson and Kjellmer 1964) by fluid shifts between various structural and/or functional compartments (Robinson 1960) Be this as it may, we feel that the present experiments are more appropriate than previously reported work for illuminating the physiological role of the alteration in membrane permeability, possibly brought about by muscular contractions We therefore conclude that this phenomenon, which is similar to the effects of insulin action, if existent at all in man, seems to play a minor role in the regulation of substrate entry into contracting muscle cells An increased uptake of glucose into working muscles (*e g* Ingle, Morley and Stetten 1955) would thus depend primarily on alterations in, *e g* local concentration gradients and intracellular structural and metabolic factors, and not on the membrane resistance

We thank Professor John Lind for a gift of d(+)xylose and Veikko Heimonen and Aimo Kuukka for their help as both experimental subjects and technical assistants

This study was aided by a grant from the Finnish Association of Sports Medicine to the working team and by a grant from the State Commission of Sports to one of the authors (J H

References

BARCLAY J A W T COOKE R A KENNEY and M E NUTT The effect of exercise on renal blood flow in man *J Physiol* (Lond) 1945 *104* 14P

BARCLAY J A W T COOKE R A KENNEY and M E NUTT The effects of water diuresis and exercise on the volume and composition of urine *Amer J Physiol* 1947 *148* 327—337

CARLIN H and O HECHTER Effects of insulin on the permeability of d and l xylose and d and l arabinose in rat diaphragm muscle *J gen Physiol* 1961 *45* 309—316

CARLSTEN A and G GRIMBERG *The circulatory response to muscular exercise in man* Pp 40—42 Charles C Thomas Springfield 1966

CHAPMAN C B A HENSCHEL, J MINCKLER A FORSGREN and A KEYS The effect of exercise on renal pla ma flo n norm [illegible]

C [illegible] following

C [illegible] *Med cal*

DOMINGUEZ, R, H GOLDBLATT and E POMERENE Kinetics of the excretion and utilization of xylose *Amer J Physiol* 1937 *119* 429—438

DRURY, D R and A N WICK Mechanism of action of insulin *Diabetes* 1955 *4* 203—206

EICHHORN J and O HECHTER Insulin induced accumulation of d xylose against an apparent concentration gradient in diaphragm muscle *in vitro* *J gen Physiol* 1961 *45* 15—22

FISCHER, R A, *Statistical methods for research workers* 11th ed, revised Oliver & Boyd Edinburgh and London 1950

GOLDSTEIN, M S Humoral character of the hypoglycemic effect of muscular work. *Fed Proc* 1960 *19* 164

GOLDSTEIN, M S, W L HENRY, B HUDDLESTUN and R LEVINE, Action of insulin on transfer of sugars across cell barriers common chemical configuration of substances responsive to the action of the hormone *Amer J Physiol* 1953 a *173* 207—211

GOLDSTEIN M S V MULLICK, B HUDDLESTUN and R. LEVINE Action of muscular work on transfer of sugars across cell barriers comparison with action of insulin *Amer J Physiol* 1953 b *173* 212—216

HELMREICH E and C F CORI, Some problems of permeability of tissue cells to sugars *Ciba Foundation Colloquia on Endocrinology Churchill, London* 1956 *9* 227—239

HELMREICH E and C F CORI Studies of tissue permeability II The distribution of pentoses between plasma and muscle *J biol Chem* 1957 *224* 668—679

JACOBSON S and I KJELLMER, Accumulation of fluid in exercising skeletal muscle *Acta physiol scand* 1964 *60* 286—292

KEITH N M M H POWER and R D PETERSON Renal excretion of xylose after intravenous injection in man *Proc Mayo Clin* 1934 *9* 43—45

KIPNIS D M and C F CORI Studies of tissue permeability III The effect of insulin on pentose uptake by the diaphragm *J biol Chem* 1957 *224* 681—693

LEVINE R. and M S GOLDSTEIN On the mechanism of action of insulin *Recent Progr Hormone Res* 1955 *11* 343—380

LEVINE, R M S GOLDSTEIN B HUDDLESTUN and S P KLEIN Action on insulin on the permeability of cells to free hexoses as studied by its effect on the distribution of galactose *Amer J Physiol* 1950 *163* 70—76

MCNEMAR Q *Psychological Statistics* 2nd ed P 268 John Wiley & Sons New York 1955

MILLER H C and D C DARROW Relation of serum and of muscle electrolyte particularly

Factors affection glucose transport in heart muscle and erythrocytes *Fed Proc* 1965 *24* 1040—1045

NELSON N J A photometric adaptation of the Somogyi method for the determination of glucose *J biol Chem* 1955 *153* 375—380

PARK C R R L POST C F KALMAN J H WRIGHT JR L H JOHNSON and H E MORGAN The transport of glucose and other sugars across cell membranes and the effect of insulin *Ciba Foundation Colloquia of Endocrinology Churchill London* 1955 *9* 240—260

R CANDELA R M RODRIGUEZ LOPEZ T CASTILLA CORTAZAR and J L R CANDELA Correlation of the effects of insulin and of muscular contraction *in vitro* on uptake of glucose by the frog *Nature* Lond 1962 *195* 712

ROBINSON J R *Body fluid dynamics* In Comar C L and F Bronner (eds) Mineral Metabolism An advanced treatise Vol I Part A pp 205 248 Academic Press 1960

SACKS J and J F SMITH Effects of insulin and activity on pentose transport into muscle *Amer J Physiol* 1958 *192* 287 289

SEGAL, S J B WYNGAARDEN and J FOLEY The effect of insulin on blood levels of infused pentose in men. *J clin Invest* 1957 *36* 1383—1394

J biol Chem 1945 *160* 69 73

J biol Chem 1952 *195* 19—23

data which reflect a continuum of exponential processes

WELT L G J ORLOFF D M KYDD and J E OLTMAN Example of cellular hyperosmolarity *J clin Invest* 1950 *29* 935—939

WYNGAARDEN J B, S SEGAL and J B FOLEY Physiological disposition and metabolic fate of infused pentoses in man *J clin Invest* 1957 *36* 1395—1407

Acta physiol. scand. 1967. 69. 187—190

From the Department of Cell Physiology, Psychiatric Clinic, St Goran's Hospital Stockholm, Sweden

The Effect of Bile Obstruction on the Oxidation Rate of Ethanol in the Rat

By

K H KIESSLING and L PILSTROM

Received 4 October 1966

Abstract

KIESSLING, K -H and L PILSTROM *The effect of bile obstruction on the oxidation rate of ethanol in the rat* Acta physiol scand 1967 *69* 187—190

In male Wistar rats the bile duct was tied up Control rats were given a sham operation After a week a single ethanol dose was given intraperitoneally and blood samples were analysed for ethanol In the rats with obstructed bile flow ethanol was oxidized at a considerably lower rate than in the controls The possible reasons for this are discussed

Many inhibitors *in vitro* of liver alcohol dehydrogenase (LADH) have been described, such as alifatic carboxylic acids (Winer and Theorell 1960) imidazole (Theorell and McKinley McKee 1961), pyrazole (Theorell and Yonetani 1963), *o*-phenantrolene (Vallee, Williams and Hoch 1959) and bilirubin (Flitman and Worth Jr 1966) The mechanisms for the action of these inhibitors have been summarized in a review by Theorell (1965) He also reports *in vitro* inhibition of LADH with pyrazole Theorell 1965) In the present paper the effect of experimentally caused bile obstruction on the oxidation rate of ethanol in rats has been studied

Experimental

Seven to-ten month-old male rats of Wistar origin were used in the experiments The rats weighing 3 0—400 g were divided into pairs making the weight of the two rats in each pair as equal as possible

Operation procedure Anesthesia was given by the intraperitoneal injection of 0 1 ml of 3 % nembutal per 100 g body weight If further anesthesia was necessary diethyl ether was given by inhalation

An incision about one and a half centimeters long was made in the ventral midline just caudally of the sternum Through this incision the duodenum was picked out and in one rat of each pair two silk ligatures were tied around the bile duct The other rat of the pair was given a sham operation by putting a ligature under the bile duct and removing it immediately The duodenum was then replaced in position and the muscle layer was sewed up with catgut and the skin with silk The whole operation procedure was performed under sterile conditions and took about a quarter of an hour

After a week the animals showed signs of bile obstruct[illegible] urine and were now treated with ethanol

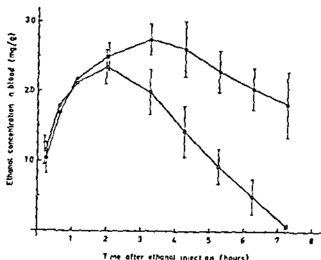


Fig 1 *Blood ethanol concentration in rats with obstructed bile flow* (●—●) *and in corresponding controls* (○—○) Each point represents the mean value of five experiments and the bars of the points correspond to the standard error of each mean value
Three hours after the ethanol treatment the difference between the ethanol concentration in the bile-obstructed rats and that in the controls is significant with $p < 0.02$ (Student's *t* test). The corresponding p values of the differences at 4 to 7 hrs after the ethanol treatment are equal to or less than 0 01

Ethanol treatment, blood sample collection and analysis 1 ml of 30 % (v/v) ethanol in physiological saline per 100 g b w was injected intraperitoneally Venous blood samples of 0 10 ml were taken from the tip of the tail every hour up to 7 hrs, beginning 15 min after the ethanol injection

The ethanol concentration of the blood samples was determined spectrophotometrically with alcohol dehydrogenase and NAD, as described by Bonnichsen (1963)

Results

The disappearance of ethanol from the sham-operated rats was faster than from those with obstructed bile flow (Fig 1) After the maximum blood-ethanol level had been reached, the rate of disappearance was 0 49 mg of ethanol/g blood per hour for the sham-operated rats The corresponding figure for the bile-obstructed rats was 0 29 mg of ethanol g blood per hour Furthermore the maximum ethanol concentration was higher in the bile-obstructed rats than in the sham operated rats

It is evident from these two facts that the oxidation rate of ethanol is impaired as a consequence of bile obstruction This was further manifested by the observation that the rats with bile obstruction needed a longer period of time to regain their balance than the sham-operated rats

Discussion

A large number of different effects of bile and bile components on enzyme activities *in vitro* and *in vivo* have been reported in the literature The hydrolysis of casein by pancreatic juice is inhibited Iizioka 1937) and the prothrombin time is prolonged by bile (Caminiti 1951 Bile salts also inhibit the activities of bile phosphatase (Biro, Grasz and Renui-Vamos 1961) and of the ATP-ase of myosin (Manni 1954) Furthermore different bile salts and sterols can act as substrates for LADH (Theorell 1965)

Besides the inhibition of LADH Flitman and Worth Jr 1966), bilirubin inhibits the activities of trypsin, chymotrypsin, amylase, intestinal alkaline phosphatase

(Bargoni and Sisini 1962, Strumia 1960) and cholesterol esterase (Kopec et al 1961) Secci and Dioguardi (1958) found that bilirubin decreases hepatic respiration This may be explained by the observations of Zetterstrom and Ernster (1956) and Ernster (1961) that bilirubin acts as a detergent on rat liver mitochondria, i e induces a loss of mitochondrial NAD and cytochrome *c*, uncouples oxidative phosphorylation, depresses the dinitrophenol induced ATP ase and stimulates the magnesium-activated ATP ase of mitochondria

These manifold effects of bile components on enzyme systems and individual enzymes makes it difficult to decide with certainty which one is repsonsible for the reduced ethanol oxidation *in vivo* (Fig 1) The fact that bilirubin inhibits LADH (*in vitro* observation) and decreases mitochondrial respiration may, however, very well explain the reduced oxidation rate of ethanol caused by experimentally produced bile obstruction The observation by Theorell (1965), that some of the bile salts act as substrates to LADH, may also be of significance in explaining our results

A reduced hepatic blood circulation as the cause of the low ethanol oxidation has been excluded by perfusion experiments A fifty per cent reduction of the blood flow through the rat liver did not significantly change the rate of ethanol disappearance from the blood (unpublished results)

This work is part of investigations made possible by grants from the Swedish Medical Science Research Council and from Nya Systemaktiebolaget Stockholm Sweden

We are indebted to Prof C H Alstrom for placing the necessary facilities at our disposal, to Miss Helga Mobius and Miss Ann Sofie Wallin for skillful technical assistance

References

BARGONI N and A SISINI Inibizione e riattivazione di idrolasi della digestione — *Boll Soc ital Biol sper* 1962 *38* 907—909

BIRO J E GRASZ and M RENUI VAMOS Die Hemmung der alkalischen Phosphatase in der Galle — *Z Vitamin Hormon u Fermentforsch* 1961 *11* 290—298

BONNICHSEN R *Ethanol Determination with Alcohol Dehydrogenase and DPN* — Methods of Enzymatic Analysis pp 285—289 Ed H U Bergmeyer Academic Press New York and London 1963

CAMINITI R Ricerche sperimentale sull attivatá protrobinica del siero Azinone della bile sul tempo di quick — *Sperimentale* 1951 *100* 517—526

ERNSTER L The mode of action of bilirubin on mitochondria Kernicterus Rep Symp IX int Congr Pediatr pp 174—192 Ed A Sass Kortsak Univ Toronto Press 1961

FLITMAN R and M H WORTH JR Inhibition of Hepatic Alcohol Dehydrogenase by Bilirubin — *J biol Chem* 1966 *241* 669—672

ITZIOKA F Über den Einfluss der Galle and die Pankreas und die Dünndarmproteolyse — *J Biochem (Tokyo* 1937 *26* 81—85

KOPEC M T DAROCHA S NIEWIAROWSKI and J STACHURSKA The antithrombin activity of glucuronic ester of bilirubin — *J clin Path* 1961 *14* 478—481

MANNI E Azione della bile e dei biliari sull attivata adenosintrifosfatasica della miosina — *Arch Sci Biol (Bologna* 1954 *38* 295—301

SECCI G C and N DIOGUARDI Influenza del tenore dietario nei riguardi dell inibizione indotta dalla bilirubina sulla respirazione endogena del fetago — *Atti Soc lombarda Sci med* 1958 253—256

STRUMIA F Effetto della bilirubina su alcuna idrolasi — *Arch Sci Biol* (Bologna 1960 *44* 337—345

THEORELL H Die Alkoholdehydrogenase Ihre Wirkungsweisen und Komplexverbindungen — *Experientia* (Basel) 1965 *21* 553—561

THEORELL, H and J S MCKINLEY MCKEE, Liver Alcohol Dehydrogenase II Equilibrium Constants of Binary and Ternary Complexes of Enzyme Coenzyme and Caprat Isobutyramide or Imidazole — *Acta chem scand* 1961

THEORELL, H. and T. YONETANI, Liver Alcohol Dehydrogenase-DPN-Pyrazole Complex. A model of a ternary intermediate in the enzyme reactions [illegible]

[illegible]

WINER, A. D. and H. THEORELL, Dissociation Constants of Ternary Complexes of Fatty Acids and Fatty Acid Amides with Horse-Liver Alcohol Dehydrogenase-Coenzyme Complexes. — *Acta chem. scand.* 1960. *14*. 1729—1742.

ZETTERSTRÖM, R. and L. ERNSTER, Bilirubin, an uncoupler of oxidative phosphorylation in isolated mitochondria. — *Nature (Lond.)* 1956. *178*. 1335—1337.

Acta physiol. scand 1967. 69 191—202
From the Department of Physiology, University of Göteborg Sweden

Suppression of Cortical Spontaneous Barbiturate Spindles via Specific and Unspecific Projection Spinal Pathways

By

SVEN A ANDERSSON

Received 4 October 1966

Abstract

ANDERSSON, S A *Suppression of cortical spontaneous barbiturate spindles via specific and unspecific projection spinal pathways*, Acta physiol scand 1967 69 191—202

The effects of peripheral adequate stimulation on cortical barbiturate spindles were investigated after spinal lesions in lightly anesthetized cats Certain effects can be evoked from the specific projection pathways the dorsal columns and the spino-cervical tract, but a generalized suppression of cortical spindles was obtained mainly via two other pathways One of these pathways identified as the bilateral ventral flexion reflex tract, ascends in the ventral funiculus The other pathway ascends in the dorsolateral funiculus and consists of thin, possibly unmyelinated fibres. Both pathways could be activated by low and high threshold receptors in bilateral fields and gave a desynchronization of cortical spindles outlasting the duration of stimulation The cortical effects mediated via the specific projection spinal pathways were mainly found in somatosensory projection areas with a local abolition of spindles and increased background activity In very light anesthesia a small and shortlasting generalized effect could sometimes be obtained

In the spinal cord the short latency projection upon the somatosensory cortex is mainly mediated via two ascending pathways, the dorsal column pathway and the spino-cervico-lemniscal pathway (Morin 1955 Andersson 1962, Norrsell and Voorhoeve 1962, Oscarsson and Rosén 1966 b) These pathways, frequently referred to as the specific projection pathways, have a topographically arranged cortical projection Via either of these pathways cells in VPL (Landgren, Nordwall and Wengstrom 1965, Andersen, Andersson and Landgren 1966) and the somatosensory areas I (Andersson and Norrsell, unpublished) and II (Andersson 1962) can be activated by peripheral stimuli, specific with regard to place and modality In preparations with the specific projection pathways transected in the spinal cord cells in cortex can still be activated via the remaining ventral spinal pathways but the effect is generalized throughout the cortex without a distinct topographical organization Furthermore, also cells in the somatosensory cortex being place and modality specific in the intact anesthetized animal, are activated via these pathways The activation of the cortical cells occur after a long delay and outlasts the stimulus for a considerable time (Andersson 1962, Andersson, Norrsell and Wolpow 1964)

In an unanesthetized preparation any adequate stimuli applied to skin can suppress the slow cortical rhythms occurring during drowsiness and light sleep Simultaneously a fast low voltage activity appears, the cortical arousal reaction This effect is mediated via the ascending reticular activating system (Moruzzi and Magoun 1949) into the unspecific thalamocortical projection system, which influences cortex in widespread areas through the anterior nuclei of the thalamus (Jasper 1960)

Originally it was supposed that the reticular activating system was a parallel system to the specific sensory system receiving collateral activation from sensory paths (Moruzzi and Magoun 1949) However, strong cortical generalized effects were obtained by peripheral adequate stimulation after section of these pathways in the spinal cord indicating that other pathways are involved in this cortical action (Andersson 1962, Andersson *et al*, 1964)

This investigation was undertaken in order to evaluate the degree of generalized cortical effect obtained by the spinal specific projection pathways in comparison with other spinal pathways Some of the results have been published in a preliminary report (Andersson 1966)

Methods

The experiments were performed on adult cats After operation under ether anesthesia the animals were given pentobarbital sodium (Nembutal) in amounts preventing spontaneous movements but allowing flexion reflexes to be elicited by pinching All wounds and points of fixation of the cat were infiltrated with a local anesthetic In some experiments gallamine triethiodide (Flaxedil) was given and the animal respired artificially

The cortex was widely exposed bilaterally and covered with mineral oil at 38 C in a pool provided by the skin Particular attention was given to keep the temperature of the animal within a normal range In most experiments the blood pressure was recorded continuously After laminectomy lesions were made in the spinal cord by means of dissection with the aid of forceps and a high power binocular dissection microscope The extent and location of the lesions are given in the Results In some experiments spinal fascicules were dissected for electrical stimulation All lesions were controlled histologically in serial sections of the parts of the cord containing the lesions In some experiments cerebellum was removed by suction

The peripheral adequate stimuli used were either light cutaneous (jets of air or brushing the hairs or strong stimuli (pinching of the skin pressure on the intact limb or forced joint movements) In some experiments electrical stimuli delivered from a stimulator (Grass S 4) were given to peripheral structures or to the dissected fascicles of the spinal cord Timed tactile stimuli could be given with a soft brush moved by an electromagnetic device The activity of the exposed cortex was recorded monopolarly against an inactive reference electrode with silver silverchloride electrodes and fed into an EEG recorder (Polygraph Model 5 Grass) In some experiments an AC-coupled preamplifier and an ordinary oscilloscope were used as well

Results

I *Effects via specific projection spinal pathways*

The effects mediated via the specific projection spinal pathways the dorsal columns (DC) and the spinocervical tract SCT) can be studied after selective lesions in the spinal cord

a *The dorsal column pathway*

In the experiment illustrated in Fig 1 all the spinal cord was interrupted at Th9 except for the DC and the dorsal part of the dorsolateral funiculus on the right

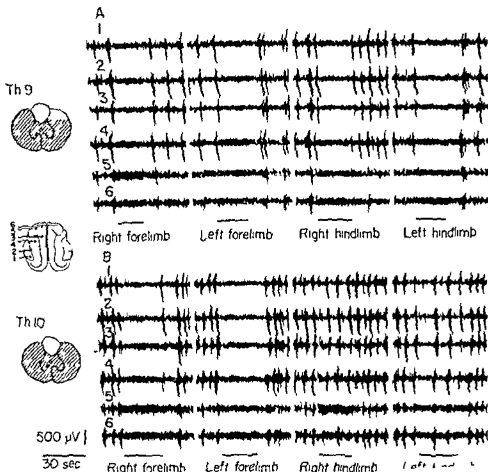


Fig 1 A Effects of ...
The eff...
funicul...
eff...
tio...
of...
sh...

side of the cord The extent of the lesion is indicated as a hatched area in the schematic cross-section of the cord The location of the recording electrodes are shown in the schematic drawing of the cortex The duration of the stimulation is indicated with a bar

Both the specific projection pathways ascend... ...abolished the cortical spindle ... a time outlasting the duration of the stimulus Since the lesion was made in the low thoracic cord the pathways from the forelimbs were intact and the effect

could be used as a control The effects obtained by stimulation of the hindlimbs could be mediated only via the part of the cord left intact by the lesion Stimulation of the hindlimbs eliminated the spindles throughout the left hemisphere as effectively as did the stimulation at the forelimb and there was no difference between the effects from the right and left limb This observation was surprising, since it is known that the specific projection pathways are crossed and have a topographically arranged projection in the somatosensory cortex In order to investigate if this effect was mediated via the dorsal columns or via the dorsolateral funiculus a second lesion was made at the level of Th10 After this lesion only the dorsal columns remained intact This lesion did not influence the response to stimulation of the forelimbs but changed completely the effects from the hindlimbs (Fig 1 B) Stimulation of the right hindlimb elicited a local effect in the somatosensory cortex (records 5 and 6) with elimination of most of the spindles and an increase in the background activity The duration of this effect was well synchronized with the duration of the stimulation An adequate stimulus given ipsilaterally with regard to the hemisphere (left hind limb) had no such effect Outside the somatosensory areas only a slight decrease in the rate of occurrence of spindles could be obtained by activation of the dorsal column pathway Usually the effects were similar as illustrated in Fig 1 B Spindles appeared during the stimulation but less frequently In some experiments the spindles were eliminated completely during the stimulation under very light anesthesia but a longlasting after effect was never obtained The cortical effects elicited via the dorsal columns were usually best obtained by a continuous light stimulation of the skin such as brushing the hair Very small or no effects were obtained by a steady stimulus such as pinching with a forceps Pupil dilatation or changes in the blood sure were not obtained via the dorsal columns

3 The spinocervical tract

The experiments with only the dorsal columns intact showed that the cortical effects obtained via this pathway were localized mainly to the somatosensory cortex The strong generalized abolition of spindles illustrated in Fig 1 A should be due to a pathway in the dorsolateral funiculus and the possibility existed that the effects were mediated via the spino-cervico-lemniscal pathway This pathway crosses the midline between C1 and C3 after its relay in the lateral cervical nucleus

The cortical effects obtained via this pathway were studied after bilateral hemisections of the spinal cord at C1 and C3 as shown in Fig 2 The dorsal columns were completely interrupted A large surface potential could be elicited in the left somatosensory cortex in reponse to a short adequate stimulus (touch) to the right hindlimb (Fig 2) showing that the pathway was intact Fig 2 shows a continuous record of cortical activity at electrode locations as indicated in the schematic drawing of the cortex *As a test of the cortical responsitivity a strong pinch* was applied to the skin on the right side of the face which gave a complete abolition of the spindles bilaterally and an increased background activity in the somatosensory cortex A similar stimulation of the right hind and forelimb had almost no effect outside the left somatosensory

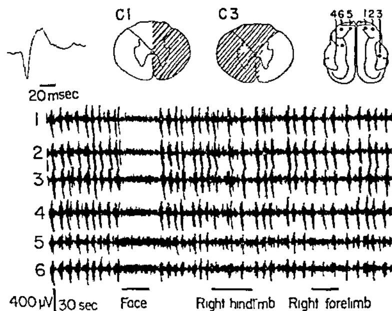


Fig. 2. Cortical effects mediated via the spino-cervico-lemniscal pathway. Above to the left a short latency potential obtained in response to an adequate tactile stimulus to the right hindlimb. The continuous recording shows the effect of pinching the right side of the face and continuous touching of the right fore- and hindlimb.

areas to which the intact pathway projected. In these areas some of the spindles were abolished, others reduced in size. The background activity increased but not to the same extent as after activation of the dorsal column pathway (Fig. 1). The duration of these effects was similar as the duration of the stimulus and longlasting after effects were never seen. Outside the area of projection the spino-cervico-lemniscal pathway gave a slight reduction in the rate of occurrence of spindles in some experiments but never a complete abolition of spindles. Also via this pathway the most pronounced cortical effects were obtained by light continuous touching of the skin. Changes in blood pressure or pupil dilatation were not found.

These experiments showed that neither the dorsal column nor the spinocervical tract mediated the strong generalized abolition of spindles illustrated in Fig. 1. It will be shown below that this effect is mediated by another pathway ascending in the dorsal part of the lateral funiculus.

II. *Effects via non specific pathways*

a. *Ventral pathways*

A lesion of the dorsal half of the spinal cord interrupts the specific projection pathways. Fig. 3 A shows the effect of pinching the left forelimb and the left hindlimb after such a lesion at Th10. The anesthesia was rather deep when the activity in Fig. 3 A was recorded. Pinching the forelimb gave only a very small effect of approximately the same duration as the stimulus. A similar stimulus applied to the left

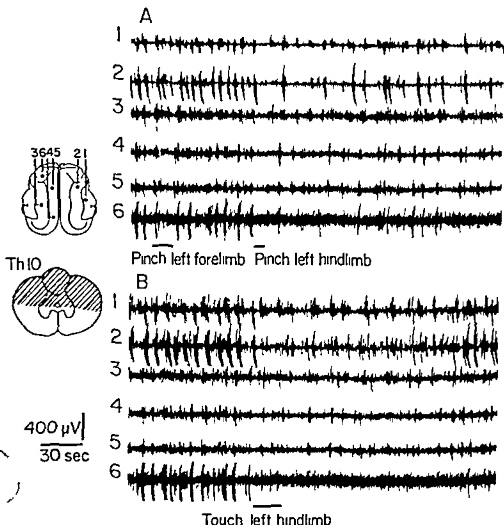


Fig 3 A Effects of pinching the left forelimb and hindlimb during relatively deep anesthesia B Effects of continuous touching the left hindlimb during light anesthesia in the same experiment

hindlimb elicited a longlasting reduction of the spindle activity bilaterally and the same effect was obtained also from the right hindlimb. Light stimuli gave no effect on the cortical activity. Later, during the same experiments in light anesthesia, a similar effect as that seen in Fig 3 A was obtained by light touch of the left (Fig 3 B) or the right hindlimb. In this condition a pronounced effect on the cortical spindles could be elicited also from the forelimbs. Apparently the ventral spinal cord contains ascending pathways capable of mediating effects that can desynchronize cortical spindles. Afferents from low threshold receptors can activate the paths. Desynchronization of the barbiturate spindles was most effectively obtained by stimulation of the skin and by forced joint movements. The extent to which muscle afferents contribute to the cortical response was not investigated.

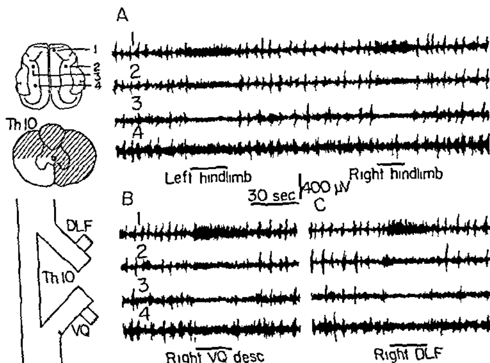


Fig 4 4 Effects of pinching the lef [illegible]

The striking difference between the effects of stimulation of the forelimbs and the hindlimbs (Fig 3 A) is most likely due to an *interruption of a descending pathway* in the dorsal part of the cord giving tonic inhibition of the transmission from the afferents to the ascending pathways A ventral pathway having the characteristics of being activated by *bilateral flexor reflex afferents* and receiving tonic inhibition of the transmission from the afferents to the ascending fibres has been described Lundberg and Oscarsson 1962) This pathway, the bilateral ventral flexion reflex tract (bVFRT) is monosynaptically excited from a pathway descending in the ventral part of the spinal cord (Holmqvist Lundberg and Oscarsson 1960a) This gave a possibility to test if bVFRT was one of the pathways through which elimination of cortical spindles could be obtained In the *experiments illustrated in Fig 4* the spinal cord was transected except for one of the ventral quadrants The dorsal columns were removed and the sectioned ventral quadrant was *dissected for stimulation* in descending direction The records in Fig 4 A show the effects obtained by pinching the left and right hindlimb A clear-cut desynchronization was obtained and the effect was very similar to that elicited by electrical stimulation (200 sec 3 V, 0 1 msec) in descending direction of the dissected ventral quadrant, showing that bVFRT mediates effects giving generalized effects in the cerebral cortex



Fig 5 Effects of pinching the forelimbs and hindlimbs The effects from the hindlimbs mediated only via the dorsal part of the right lateral funiculus

b *Effects via dorsal pathways*

In Fig 4 C is shown that electrical stimulation of the dissected right dorsolateral funiculus gave a bilateral effect on the cortical barbiturate spindles similar to that elicited by stimuli applied to the hindlimbs and to activation of the bVFRT via the descending pathway The maximal effect was obtained with rather low frequencies (20/sec) and only with long duration and high voltage of the shocks (20 V, 3 msec) This indicates that the effect was mediated by thin ascending fibres The effects of adequate peripheral stimulation is shown in Fig 5 At the level of Th9 the spinal cord was transected except the most dorsal part of the right dorsolateral funiculus The dorsal columns and the left half of the spinal cord were interrupted in C1 This lesion eliminates the spino cervico lemniscal pathway, ascending in the dorsolateral funiculus, and crossing the midline below C1 Pinching the right and left hindlimb gave an effect on the cortical activity similar to that obtained bilaterally from the forelimbs During very light anesthesia a strong cortical effect was elicited also by light stimuli indicating that low threshold receptors contribute in the activation of this pathway The recordings shown in Fig 6 were taken in an experiment with only a small part of the right dorsolateral funiculus intact at Th10 and the left half of the cord and most of the dorsal columns interrupted at C1 Pinch of the left (Fig 6 A) or right forelimb elicited a longlasting elimination of the spindles A similar pinch of the left Fig 6 C) or right hindlimb was equally effective However, also a continuous light touch of the right Fig 6 B) or left hindlimb gave a pronounced effect and the spindles were eliminated for a considerable time The light anesthesia in this preparation is indicated by the reduced background activity during the early phase of stimulation This pathway seems to be activated mainly from the skin Forced joint movements were less effective than pinching of the skin The effects of adequate stimulation of exposed subcutaneous structures were not tested The cortical effects were usually of long duration outlasting the period of stimulation for a considerable time, also in somewhat deeper anesthesia Particularly during light

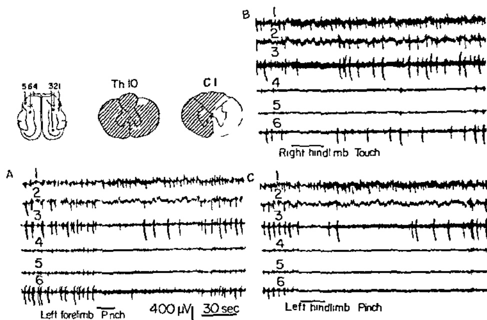


Fig 6 *A* Effects of pinching the left forelimb *B* Effects of continuous touching the right hindlimb *C* Effects of pinching the left hindlimb The effects from the hindlimbs mediated via the dorsal part of the right dorsolateral funiculus

anesthesia the cortical desynchronization was accompanied by a bilateral pupil dilatation and a rise in the blood pressure Also these effects were obtained from bilateral fields The pupil dilatation and the elimination of the cortical spindles were parallel in time The rise in blood pressure occurred considerably later

In order to exclude that some of the effects were mediated through the dorsal spinocerebellar tract (DSCT) and cerebellar structures the cerebellum was removed in addition to similar spinal lesions as shown in Fig 3 and 4 After removal of cerebellum the effect of peripheral stimulation remained unchanged

The exact location of the pathway mediating these effects could not be determined by the lesions used in the present experiments but a large number of fibres apparently ascend in the most dorsal part of the dorsolateral funiculus In some experiments marked cortical effects were found after a lesion in the low thoracic cord sparing only a thin and narrow bridge lateral to the dorsal column (see Fig 5 and 6 A second lesion, including this part of the cord a few millimeters above or below the bridge abolished completely the effect of stimulation of the hindlimbs

Discussion

In the present investigation elimination of cortical spontaneous barbiturate spindles were used as an index of the ability of various pathways to induce the cortical arousal reaction It has been assumed that the barbiturate spindles have a similar genesis as the sleep spindles appearing in the intact unanesthetized animal during

drowsiness and light sleep (Bremer 1958) The sleep spindles are easily abolished by light peripheral stimuli Such peripheral stimuli can induce a longlasting desynchronization of the spindles also during light barbiturate anesthesia and there are no reasons to believe that there are different mechanisms mediating the desynchronization of cortical spindles during the two conditions

Spinal pathways mediating generalized cortical effects were found in both the ventral and the dorsal parts of the cord The ventral pathway could be identified as the bilateral ventral flexion reflex tract (bVFRT) (Lundberg and Oscarsson 1962) This pathway is activated by the flexor reflex afferents (FRA) group II and III muscle afferents cutaneous and high threshold joint afferents Recently the bVFRT was found to be the spinal component of a spinoreticulo cerebellar path with a relay in the lateral reticular nucleus (Grant Oscarsson and Rosén 1966) The effects in the cerebral cortex via bVFRT show that this pathway also has other important connections The effects in the cerebral cortex are presumably exerted via the ascending reticular activating system and the unspecific thalamocortical system (Lorento de Nó 1949 Jasper 1960) As pointed out by Oscarsson and Rosen (1966 a) the similarity in the termination of the fibres in this system and of the cerebellopetal fibres arising from the lateral reticular nucleus is interesting The last mentioned fibres make contacts with superficial granule cells with termination on the distal branches of the Purkinje dendrites (Szentágothai 1964) The unspecific thalamocortical fibres seem to exert their action predominantly on the distal branches of the dendrites (Lorento de No 1949 Jasper 1960 Nacimiento Lux and Creutzfeldt 1964) These types of termination can be important in the modification of the background activity in both the cerebral and the cerebellar cortices

The pathway in the dorsal part of the lateral funiculus giving cortical desynchronization will be analyzed further in a separate communication This pathway cannot be identified with either the dorsal spinocerebellar tract or the spinocervical tract ascending in this part of the cord These pathways are activated almost exclusively by ipsilateral afferents but the pathway giving generalized cortical effects was excited from bilateral fields Although the latency could not be determined by the methods used in the present experiments it was in the order of seconds indicating that the effects were mediated via a very slowly conducting system High voltage and long duration of the single shocks were required for electrical excitation of the pathway and presumably the pathway consists of thin possibly unmyelinated fibres Preliminary experiments have shown that electrical stimuli of a strength exciting unmyelinated fibres in the peripheral nerve elicit a late mass discharge in the dissected dorsolateral funiculus in the low thoracic cord This potential could be obtained from skin and mixed nerves of both hindlimbs but not from muscle nerves A large number of unmyelinated fibres are found in the dorsal part of the lateral funiculus Ranson 1913 Via such fibres in this part of the cord Evans (1961) obtained dilatation of the pupils by electrical stimulation of unmyelinated fibres in skin nerves bilaterally A rise in the blood pressure can also be obtained via ascending fibres in the dosolateral funiculus Johansson 1962

It is interesting that this dorsal pathway is activated by afferents from bilateral fields In an extensive series of investigations in different species Oscarsson (1964) found that pathways ascending in the dorsal part of the spinal cord were almost exclusively activated by ipsilateral afferents Contralateral and bilateral activation was found only in pathways ascending in the ventral parts of the cord This classification is apparently not valid for ascending pathways with small diameter fibres

The main cortical effects mediated via the specific projection spinal pathways were localized to the somatosensory projection areas However, in very light anesthesia a generalized elimination of spindle bursts could be obtained during the time of stimulation The striking difference between this effect and the longlasting effect by stimulation of the unspecific spinal pathways do not support the hypothesis that the ascending activating system receives its main inflow by collaterals from the specific projection system (Moruzzi and Magoun 1949) Neither is there any anatomical support for connection between the medial lemniscus and the reticular formation (Matzke 1951, Rossi and Brodal 1957, Bowsher 1958) The present investigation shows that activation of pathways outside the specific projection system can give a generalized cortical desynchronization and that this effect can be obtained by light as well as by strong stimuli via either the ventral or the dorsal pathway These pathways are more sensitive to anesthesia than the specific projection pathways In the anesthetized preparation polysynaptic transmission would be partially blocked and a more massive activation of the afferents is necessary than in the unanesthetized animal Of great importance for the transmission from afferents to the ascending fibres are also the effects of descending control systems (Holmqvist, Lundberg and Oscarsson 1960 b) but there is no information how such systems operate in an intact unanesthetized animal

This work was supported by the Swedish Medical Research Council (Project No B67 14X 55-03)

References

ANDERSEN P, S A ANDERSSON and S LANDGREN Some properties of the thalamic relay cells in the spino-cervico-lemniscal path *Acta physiol scand* 1966 *68* 72–83

ANDERSSON S A Projection of different spinal pathways to the second somatic sensory area in cat *Acta physiol scand* 1962 *56* Suppl 194

ANDERSSON S A Intracellular postsynaptic potentials in the somatosensory cortex of the cat *Nature* (Lond 1965 *205* 297—298

ANDERSSON S A Suppression of cortical spontaneous barbiturate spindles via spinal pathways *Acta physiol scand* 1966 *68* Suppl 277

ANDERSSON S A L NORRSELL and E R WOLPOW Cortical synchronization and desynchronization via spinal pathways *Acta physiol scand* 1964 *61* 144—158

BOWSHER D Projection of the gracile and cuneate nuclei in *Macaca Mulatta* an experimental degeneration study *J comp Neurol* 1958 *110* 135—155

BREMER F Cerebral and cerebellar potentials *Physiol Rev* 1958 *38* 357—388

EVANS M H The spinal pathways of the myelinated and the non myelinated afferent nerve fibres that mediate reflex dilatation of the pupils *J Physiol* Lond 1961 *158* 560—572

GRANT G O OSCARSSON and I ROSÉN Functional organization of the spinoreticulocerebellar path with identification of its spinal component *Exp Brain Res* 1966 *1* 306—319

HOLMQVIST B A LUNDBERG and O OSCARSSON A supraspinal control system monosynaptically connected with an ascending spinal pathway *Arch ital Biol* 1960a *98* 402—422

HOLMQVIST, B, A LUNDBERG and O OSCARSSON, Supraspinal inhibitory control of transmission to three ascending spinal pathways influenced by the flexion reflex afferents *Arch ital Biol* 1960b *98* 60—80

JASPER, H H, Unspecific thalamocortical relations In *Handbook of Physiology*, Ed J Field, William and Wilkins Co 1960, Sec *I II* 1307—1321

JOHANSSON, B, Circulatory responses to stimulation of somatic afferents *Acta physiol scand* 1962 *57* Suppl 198

LANDGREN, S, A NORDVALL and C WENGSTROM, The location of the thalamic relay in the spino-cervico lemniscal path *Acta physiol scand* 1965 *65* 164—175

LORENTE DE NO, R, Cerebral cortex architecture, intracortical connections, motor projections In *Physiology of the Nervous System* (3rd ed), ed by J F Fulton, New York and London Oxford 1949 ch 15

LUNDBERG, A and O OSCARSSON, Two ascending pathways in the ventral part of the cord *Acta physiol scand* 1962 *54* 270—286

MATZKE, H A, The course of fibres arising from the nucleus gracilis and cuneatus of the cat *J comp Neurol* 1951 *94* 439—452

MORIN, F, A new spinal pathway for cutaneous impulses *Amer J Physiol* 1955 *183* 245—252

MORUZZI, G and H MAGOUN, Brain stem reticular formation and activation of the EEG *Electroenceph clin Neurophysiol* 1949 *1* 455—475

NACIMIENTO, A C, H D LUX and O D CREUTZFELDT, Postsynaptische Potentiale von Nervenzellen des motorischen Cortex nach elektrischer Reizung spezifischer und unspezifischer Thalamuskerne *Pflugers Arch ges Physiol* 1964 *281* 152—169

NORRSELL, U and P VOORHOEVE, Tactile pathways from the hindlimb to the cerebral cortex in cat *Acta physiol scand* 1962 *54* 9—17

OSCARSSON, O, Differential course and organization of uncrossed and crossed long ascending spinal tracts In Physiol of Spinal Neurons, Ed J C Eccles and J P Schadé *Prog Brain Res* 1964 *12* 164—178

OSCARSSON, O and I ROSÉN, Response characteristics of reticulocerebellar neurones activated from spinal afferents *Exp Brain Res* 1966 a *1* 320—328

OSCARSSON, O and I ROSEN Short latency projections to the cat's cerebral cortex from skin and muscle afferents in the contralateral forelimb *J Physiol* (Lond) 1966 b *182* 164—184

RANSON, S W, The course within the spinal cord of the nonmedullated fibres of the dorsal roots a study of Lissauer's tract in the cat *J comp Neurol* 1913 *23* 259—281

ROSSI, G F and A BRODAL Terminal distribution of spinoreticular fibres in the cat *Arch Neurol Psychiat* (Chicago) 1957 *78* 439—453

SZENTAGOTHAI, J Anatomical aspects of junctional transformation In *Information Processing in the Nervous System* pp 119—136 (ed R W Gerard) Amsterdam Exerpta Medica 1964

Acta physiol. scand. 1967. 69. 203—208

From the Institute of Nutrition Research and Institute for Zoophysiology University of Os
Blindern, Norway

Carbon Dioxide Excretion and pH-Variations in Diving Ducks after Carbonic Anhydrase Inhibition

By

HARALD T ANDERSEN and BO-EGIL HUSTVEDT

Received 18 October 1966

Abstract

ANDERSEN, H T and B-E HUSTVEDT *Carbon dioxide excretion and pH-variations in diving ducks after carbonic anhydrase inhibition* Acta physiol scand 1967 69 203—208

Acetazolamide (Diamox®) was administered intravenously to domestic ducks in doses ... mg kg b w The smallest dose of acetazolamide required for ... anhydrase in the duck is approx ... no decrease ... ve ... du ... afte ... Th ... to ... red ... the arterial total CO_2 ... (10—+13) The excretion of CO_2 ... during which the ... capacity in the blood ... the arterial total CO_2 from approximately 25 meq/l to approximately 15—20 meq/l whereas it normally falls to about 9—10 meq l upon emersion

Experiments on dogs and rats have shown that inhibition of carbonic anhydrase by 2 acetylamino-1,3,4-thiodiazol-5-sulfonamide (acetazolamide) causes transient hyperventilation and a fall in the alveolar partial pressure of carbon dioxide (P_{CO_2} with concomitant retention of CO_2 as revealed by increasing P_{CO_2} in arterial and venous blood and in the tissues Tomashefski *et al* 1954 Mithoefer and Davis 1958, Mithoefer 1959) A new balance between CO_2 production and excretion is attained 30 –60 min after i v injection of acetazolamide Mithoefer 1959) at which interval of time the inhibitory effect is maximal (Mithoefer and Davis 1958) at which interval period the capacity for transport of CO_2 is presumably greatly reduced During this interesting therefore to impose an acute stress on the system by loading it with CO_2 to be eliminated via the lungs Such an experimental approach is not feasible unless the subjects tolerate severe hypercapnia For this reason we have chosen to study an avian diver the domestic duck, which may endure submersion asphyxia for as much as 15 min Andersen 1959 a Also, renal excretion of bicarbonate is not

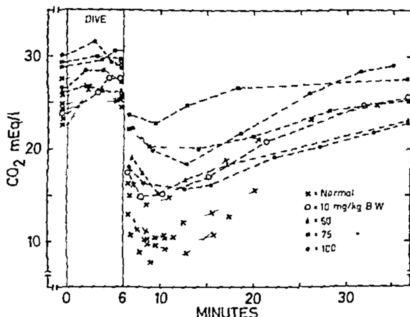


Fig 3 Total CO_2 before during and after 6 minutes of submersion in intact ducks and in ducks given acetazolamide (Lines between last symbol in diving period and first symbol in recovery period are omitted)

... sed buffering capacity in ducks injected with the inhibitor The transient ... in the arterial pH of intact birds may be explained as follows At the end of a 6 min period of diving the pH is low, the arterial P_{CO_2} is approximately doubled and the total CO_2 normal or somewhat elevated (*cf* Andersen, Hustvedt and Løvø 1965) As soon as ventilation starts CO_2 is blown off at a very rapid rate and a recovery of the arterial pH starts However nonvolatile acids particularly lactic acid, which have been formed in anaerobic tissues during the period of submersion enter the circulation and force a new drop in the arterial pH (*ibid*) The initial rapid excretion of carbon dioxide seems impossible in ducks in which the carbonic anhydrase activity has been much lowered by acetazolamide and no early recovery of the arterial pH takes place in these birds upon emersion

Total CO_2

Changes in total CO_2 before, during and after 6 minutes of submersion are shown in Fig 3 The values obtained from intact ducks and from ducks given acetazolamide are distinctly different Birds in which the carbonic anhydrase activity was inhibited displayed an elevated total CO_2 throughout the experiment This characteristic was particularly conspicuous after the underwater exposure

Because of the wide variation in pre-diving values for carbon dioxide (Fig 3) the data are not readily comparable as to carbon dioxide transport in the recovery

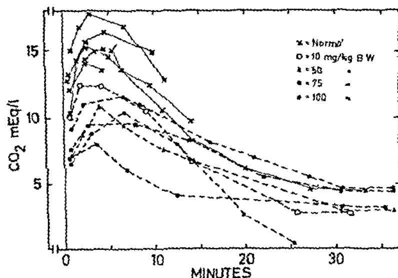


Fig. 4. Pulmonary excretion of carbon dioxide after 6 minutes of submersion in intact ducks and in ducks given acetazolamide. See text for explanation.

period. In order to make such a comparison feasible, the data has been replotted in Fig. 4. Each point shown in Fig. 4 represents the difference between the concentration of total CO_2 in plasma at the end of submersion and the concentration of total CO_2 at the time when the sample was withdrawn. This difference is a measure for the carbon dioxide excretion during post-diving hyperventilation. In intact ducks carbon dioxide elimination via the lungs reached a maximum value 2—6 min after emersion and ranged from 14.1—17.3 meq/l. The corresponding values observed in birds injected with acetazolamide were consistently lower than those of intact ducks. Also, the difference between the concentration of total CO_2 at the end of the dive and the minimum value recorded during post diving hyperventilation fell with increasing dose of acetazolamide. Thus, when 50—100 mg/kg b.w. had been administered the CO_2 excretion was only 50—60 per cent of that of intact birds.

On the basis of the data presented in this paper it is reasonable to conclude that inhibition of carbonic anhydrase by acetazolamide reduces the capacity for transport of carbon of carbon dioxide in duck blood and that the buffering capacity is similarly decreased. These changes are particularly noticeable when an increased load is put on the carbon dioxide transport system by elevating the substrate level. Our data are consistent with those of Stromme and Fog (1962) who imposed a different demand for carbon dioxide transport, hyperventilation, on human subjects to whom acetazolamide had been administered.

This study was supported by a gr... t to ... Nat... of ... der

References

ANDERSEN, H T, Depression of metabolism in the duck during experimental diving *Acta physiol scand* 1959 a *46* 234—239
ANDERSEN, H T, A note on the composition of alveolar air in the diving duck *Acta physiol scand* 1959 b *46* 240—243
ANDERSEN, H T, Factors determining the circulatory adjustments to diving I Water immersion *Acta physiol scand* 1963 *58* 173—185
ANDERSEN, H T, Stresses imposed on diving vertebrates during prolonged under water exposure In *Homeostasis and Feedback Mechanisms, Symp No XVIII Soc Exptl Biol* G M Hughes (Ed) London Cambridge Univ Press, 1964 109—127
ANDERSEN, H T, B E HUSTVEDT and A LOVO, Acid base changes in diving ducks *Acta physiol scand* 1965 *63* 128—132
MITHOEFER, J C Inhibition of carbonic anhydrase Its effect on carbon dioxide elimination by the lungs *J appl Physiol* 1959 *14* 109—115
MITHOEFER, J C and J S DAVIS, Inhibition of carbonic anhydrase Effect on tissue gas tensions in the rat *Proc Soc exp Biol* N Y 1958 *98* 797—801
STRØMME, J H and J FOG, Effect of acetazolamide on respiratory gas exchange during hyperventilation in man *J appl Physiol* 1962 *17* 6—8
SYKES, A H, Submersion anuria in the duck *J Physiol* (Lond) 1966 *184* 16P
TOMASHEFSKI, J F, H I CHINN and R T CLARK, JR, Effect of carbonic anhydrase inhibition on respiration *Amer J Physiol* 1954 *177* 451—454

Acta physiol. scand. 1967. 69. 209—212

From the Department of Anatomy, University of Helsinki, Helsinki, and from the Research Laboratory of the Sauna Seura, Helsinki, Finland

Effect of Circulatory Disturbance of the Testis on the Rectum-Testis Temperature Difference in the Rat

By

MARTTI KORMANO

Received 19 October 1966

Abstract

KORMANO, M *Effect of circulatory disturbance of the testis on the rectum testis temperature difference in the rat* Acta physiol scand 1967 *69* 209—212

Circulatory disturbance in the rat testis was produced by injecting 0 03 mmoles/kg cadmium chloride subcutaneously or by ligating the vessels of one testis Temperatures were recorded with thermocouple needles without anaesthesia Neither acute nor chronic cadmium damage to the testes, nor even vascular ligation, significantly changed the normal temperature difference between rectum and testis The reactions to heating and cooling of ligated and normal testes were very similar It is suggested that the vascular mechanisms in the adult rat testis prevent excessive heat from being carried into the testis by the internal spermatic artery, but that the scrotum as such is capable of maintaining the testicular temperature low enough in the absence of arterial blood flow

The temperature of the rat testis is known to be about 3—4° C lower than that of the rectum (Elfving 1950, Maekawa *et al* 1965, Kormano 1967) In a previous study it was observed that the temperature difference in the rat reaches the adult value at about 35 days after birth (Kormano 1967) At this age changes occur in the microvascular bed of the rat testis (Kormano, to be published), and the scrotum grows in response to testicular enlargement (Selye 1943) According to Andrews (1940), the tunica dartos muscle becomes functionally mature at the age of 9 weeks The relative importance of the scrotum and the testicular vasculature in maintaining the temperature difference during experimental circulatory disturbance is studied in the present paper

Material and methods

The material consisted of 10 ad [illegible]
ceived a sin. [illegible]
temperature [illegible]
The other 6 [illegible]

TABLE I Effect of cadmium injury of the testis on the rectum testis temperature difference in the rat.

Time after cadmium injection	Number of animals	Mean rectal temperature[1] °C	SD	Mean rectum testis difference °C	SD
Controls[2]	9	37 32	0 29	3 47	0 38
2—8 days	9	37 18	0 37	4 08	0 84
2—3 months	6	37 08	0 45	3 21	0 76

[1] Only values obtained with rectal temperature between 36 6—37.8 without external heating are included

[2] Kormano, M (1967)

TABLE II Temperature differences between rectum and testis after vascular ligation in one testis in the rat.

Number of animals	Mean rectal temperature[1] C	SD	Mean difference rectum normal testis C	SD	Mean difference rectum/ligated testis C	SD	Normal testis — ligated testis C	SD	P <
4	37 44	0 29	3 11	0 47	3 69	0 93	— 0 57	0 68	

[1] Only values obtained with rectal temperatures between 36 6—37 8 without external heating are included

being made 2—3 months after the injection In 4 animals the vessels in the pampiniform plexus were ligated just above the cranial pole of one testis a few minutes before temperature measurements were begun A detailed description of the methods for temperature registration has been given in another paper Kormano 1967

Results

The temperature differences in both acute and long-term cadmium experiments are presented in Table I Even in the acute experiment, the temperature difference was not significantly altered The group acutely damaged by cadmium presented a haemorrhagic necrosis of the testes caput epididymides and pampiniform plexuses The size of the testes in this group was about normal The scrota were swollen and contained some haemorrhagic exudate In the long-term group on the other hand, the testes were small pale and hard

Table II presents the observations on the temperature difference after ligation of the pampiniform plexus In these animals experimental cooling and heating of the testes was carried out after recording of the normal gradient to correlate the two testes In Fig 1 a typical temperature curve can be seen In spite of the ligature in one testis, the curves for the two testes did not differ much in their course except for the somewhat slower spontaneous rewarming of the ligated testis after experimental cooling with ether see Fig 1) No statistically significant difference was found between the temperatures of the two testes in this group

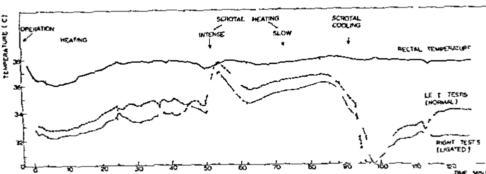


Fig 1 Record of rectal and testicular temperatures in an adult rat with a vascular ligature in the right testis Note the very similar temperatures in the two testes during the course of the experiment Rewarming of the ligated testis at the end of the experiment is somewhat slower

Discussion

Cadmium injection is a simple and certain method of damaging the testicular vasculature without surgical interference There is already complete necrosis of the testes as little as 2 days after a dose of 0 03 mmoles/kg cadmium chloride (Mason *et al* 1964), and no regeneration occurs during the first 8 days The acute cadmium group was similar to the ligated animals and these can be discussed together from the circulatory point of view (Niemi and Kormano 1965) The recordings of the animals did not show any significant differences Strong regeneration of the testicular vasculature is visible 2—3 months after cadmium injury, although the testes themselves remain small The results of the long term cadmium studies, controlled by using surgical ligature technique, showed a normal rectum testis temperature difference

In the current literature, there is convincing evidence for a counter current thermoregulatory system in the pampiniform plexus (for references see Kormano 1967) Vascular deprivation of the testis, however, did not in the present study produce any significant change in the temperature, as compared with the normal testis of the same rat Maekawa *et al* (1965) recently reported similar results However, their suggestion that cadmium does not inhibit the thermo-regulatory function of the pampiniform plexus seems erroneous The observations presented here suggest that the rat scrotum as such is capable of maintaining the low testicular temperature, and that the role of the special arrangement of the testicular vessels, in so far as they are concerned in the cooling mechanisms, is only to prevent blood from bringing such excessive amounts of heat into the testis as would overburden the cooling capacity of the scrotal sac When, after injection af cadmium, there is practically no blood entering the rat testis (Waites and Setchell 1966) and after vascular ligation definitely none, no significant diminution in the rectum — scrotal testis temperature difference follows

The present results do not exclude the possibility that the rectum-testis temperature difference cannot be maintained, if the venous part of the pampiniform plexus were selectively affected without simultaneous interference with the arterial blood flow

In fact, observations on dog (Dahl and Herrick 1959) and human (Meyhöfer and Wolf 1960) testes suggest marked elevation of the testicular temperature, if it is only the venous outflow from the testis that is disturbed

This study has been supported by a grant from Yrjo Jahnsson's Foundation

References

ANDREWS F N, Thermo regulatory function of rat scrotum I Normal development and effect of castration *Proc Soc exp Biol (N Y)* 1940 *45* 867—869

DAHL, E V and J F HERRICK, A vascular mechanism for maintaining testicular temperature by counter current exchange *Surg Gynec Obstet* 1959 *108* 697—705

ELFVING, G, Effects of the local application of heat on the physiology of testis An experimental study on rats T A Sahalan Kirjapaino Oy, Helsinki 1950 104 pp

KORMANO, M, Development of the rectum-testis temperature difference in the postnatal rat *J Reprod Fertil* 1967 in press

MAEKAWA, K, Y TSUNENARI and Y YAMAMURA, Effect of cadmium administration on intratesticular temperature in the rat *Zool Mag* 1965 *74* 170—173

MASON, K E, J A BROWN, J O YOUNG and R R NESBIT, Cadmium-induced injury of the rat testis *Anat Rec* 1964 *149* 135—147

MEYHOFER, W and J WOLF, Varicocele und Fertilitat *Derm Wschr* 1960 *142* 1116—1121

NIEMI M and M KORMANO, An angiographic study of cadmium induced vascular lesions in the testis and epididymis of the rat *Acta path microbiol scand* 1965 *63* 513—521

SELYE, H, FACTORS influencing development of scrotum *Anat Rec* 1943 *85* 377—385

WAITES, G M H and B P SETCHELL, Changes in blood flow and vascular permeability in the testis, epididymis and accessory glands after the administration of cadmium chloride *J Endocr* 1966 *34* 329—342

Acta physiol. scand. 1967. 69. 213—217

From the Metabolic Division, Department of Pharmacology, University of Gothenburg, Sweden

The Effect of Adrenaline on the Carbohydrate Metabolism in Striated Muscle

By

ANDRAS BEVIZ, ELLA MOHME-LUNDHOLM and NILS SVEDMYR

Received 21 October 1966

Abstract

BEVIZ, A., E. MOHME LUNDHOLM and N. SVEDMYR. The effect of adrenaline on the carbohydrate metabolism in striated muscle. Acta physiol. scand. 1967. 69. 213—217.

Adrenaline increased the concentration of, and changed the relationship between different hexose phosphates in isolated rat diaphragm. This indicated that adrenaline did not only influence the carbohydrate metabolism in striated muscle by activating the enzyme phosphorylase and stimulating the glycogenolysis. An increased glycogenolysis was probably the most significant effect of adrenaline but an activation of the reactions glucose-1 phosphate ⟶ glucose-6-phosphate, fructose-6-phosphate + ATP ⟶ fructose 1,6-phosphate + ADP also seemed to be of importance.

Cori and Sutherland (1951) found that in the breakdown of liver glycogen to glucose the reaction glucose + H_3PO_4 $\xrightarrow{\text{phosphorylase}}$ glucose-1-phosphate was rate-limiting. Adrenaline stimulated the breakdown of liver glycogen to glucose by increasing the concentration of active phosphorylase. Sutherland (1952 a) later showed that adrenaline in skeletal muscle also increased the phosphorylase activity by converting phosphorylase *b* ⟶ *a*. Phosphorylase *b* is only active at an AMP concentration that is not normally found in the muscle cells, while phosphorylase *a* is active in the absence of AMP. It has since been considered that the effect of adrenaline on the carbohydrate metabolism in skeletal muscle can be ascribed to this phosphorylase activation (Sutherland and Rall 1960).

Facts have emerged, however, which indicate that phosphorylase activation is not the only point of attack of adrenaline in the carbohydrate metabolism of skeletal muscle. Sutherland (1952 b) observed that adrenaline reduced the content of glucose-6-phosphate in rat diaphragm under conditions when phosphorylase activation could be excluded. Lyon and Porter (1963) found that in a certain strain of mou

se, adrenaline caused little or no increase in the content of phosphorylase *a*, but at the same time had a marked glycogenolytic and lactate increasing effect Even in Mc Ardle's disease, which is a congenital muscular disease in which the enzyme phosphorylase is lacking, adrenaline still increased the content of lactic acid in the blood (Mc Ardle 1951, Schmid and Mahler 1959)

We have attempted to localize the other possible points of attack of adrenaline in the carbohydrate metabolism of skeletal muscle by determining the concentration of intermediary metabolites *A priori* it seemed likely that adrenaline might influence some rate-limiting reaction or reactions in the glycogenolytic chain It seemed reasonable to assume that during a "steady state" the concentration of intermediary metabolites would be relatively high before this enzymatic "bottle neck", but low after it After adrenaline a change should occur, such that the concentration decreased before the rate limiting reaction, and rose after it (Krebs and Kornberg 1957)

We determined the concentration of the following metabolites in the glycogenolytic chain

glycogen ⟶ glucose-1 phosphate ⟶ glucose 6 phosphate ⟶ fructose 6-phosphate ⟶ fructose-1 6 phosphate ⟶ dihydroxiacetonphosphate ⟶ lactic acid The results obtained indicate that adrenaline influences not only the glycogen ⟶ glucose-1 phosphate reaction but also the glucose 1-phosphate ⟶ glucose 6 phosphate and possibly the fructose 6 phosphate ⟶ fructose 1 6 phosphate reaction

Method

For the experiments with determination of the glycogenolysis and the rate of lactate production in [illegible] had been starved [illegible]

[illegible] tion of 4 10 g ml At the end of the experiment the glycogen and lactic acid contents of the muscle were analysed as before and the lactic acid content of the incubation solution was also determined The lactate production was calculated from the sum of the lactic acid contents of the muscle and incubation solution at the end of the experiment less the content of the muscle at the beginning of the experiment The magnitude of the glycogenolysis was determined from the difference in the glycogen content of the muscle before and after the incubation

In the experiments with determination of the concentration of hexose phosphates each diaphragm was divided into two parts Each part was first incubated for 30 min in 15 ml Krebs Henseleit bicarbonate solution without glucose Adrenaline in a concentration of 4 10 g ml was then added to one part of the pooled diaphragm specimens After 7 5 min both pieces of muscle were frozen with freon 11 and after weighing in tared tubes were homogenized with 8 volumes [illegible] perchloric [illegible] to Hohorst (1962) Fructose-1-6-diphosphate and dihydroxiacetonphosphate were [illegible] according to Bucher and Hohorst (1962

TABLE I The effect of adrenaline on glycogenolysis and lactic acid production of isolated rat diaphragm Mean of 12 tests ± S.E.M

Glycogen	Change of glycogen content	
basal content mg per 100 g	control mg per 100 g	adrenaline mg per 100 g
137.5 ± 17.1	+7.8 ± 12.8	—49 ± 11.3 P < 0.01
Difference control — adrenaline		56.8 ± 9.0 mg per 100 g P < 0.001

Lactic acid	Production of lactic acid	
basal content mg per 100 g	control mg/100 g/15 min	adrenaline mg/100 g/15 min
30.4 ± 3.4	21.3 ± 3.4	46.3 ± 6.6
Difference adrenaline-control		25.0 ± 5.4 P < 0.01

TABLE II The effect of adrenaline on the concentration of hexose-phosphates in rat diaphragm. Mean of 6—9 tests ± S.E.M Concentration in μmoles g wet weight Glucose 1 phosphate = G 1 P, Glucose-6-phosphate = G-6-P Fructose 6-phosphate = F 6-P Fructose 1-6-phosphate = F 1-6-P Dihydroxyacetonphosphate = DAP

	G-1 P	G 6-P	F-6-P	F 1 6-P	DAP
Basal values	0.042 ± 0.004	0.185 ± 0.063	0.085 ± 0.009	0.384 ± 0.052	0.059 ± 0.03
Adrenaline	0.060 ± 0.007	0.495 ± 0.043	0.112 ± 0.007	0.743 ± 0.123	0.095 ± 0.04
Increase μmoles g after adrenaline	0.018 ± 0.005 P 0.02	0.312 ± 0.042 P < 0.001	0.027 ± 0.008 P 0.02	0.359 ± 0.138 P 0.05	0.036 ± 0.01 P 0.02
Increase per cent after adrenaline	43 ± 12	182 ± 37	33 ± 13	95 ± 23	82 ± 23

Difference (per cent) in increase between

glucose-6-phosphate and glucose 1 phosphate	139 ± 38	P 0.01
glucose 6-phosphate and fructose-6-phosphate	144 39	P 0.01
fructose 1 6-phosphate and fructose 6-phosphate	58 ± 26	P 0.0

Results

The magnitude of glycogenolysis and lactic acid production after adrenaline is shown in Table I No definite effect on the glycogenolysis was observed in the control experiments while adrenaline stimulated it by 59.6 ± 9.0 mg per 100 g The lactic acid produc

tion increased twice as much after adrenaline as in the control experiments, this increase being 22 3 mg/100 g/15 min It was estimated that 37 per cent of the disappearing glycogen was converted to lactic acid under the influence of adrenaline

The effect of adrenaline on the content of hexosephosphates in the diaphragm is shown in Table II All metabolites increased after adrenaline, these increases being statistically significant The percentage increase over the basal value has also been calculated for the different metabolites in Table II As can be seen, this percentage increase varied greatly, and statistical differences were noted in the increase of the metabolic concentration in all stages of the reaction chain glucose-1-phosphate ⟶ glucose-6-phosphate ⟶ fructose-6-phosphate ⟶ fructose-1-6 phosphate

Discussion

At enzymatic equilibrium in the glucose-1-phosphate $\xrightarrow{\text{phosphoglucomutase}}$ glucose 6 phosphate reaction, there is 5 5 per cent glucose-1-phosphate and 94 5 per cent glucose-6-phosphate, i e $\frac{\text{(G-1-P)} \ 100}{\text{G-1-P} + \text{G-6-P}} = 5\ 5$ (Najjar 1955) In untreated rat diaphragm we *found that this quotient was higher, viz 18 7 ± 1 0 per cent (n = 6), which is* to be expected in view of the assumption that a continuous conversion of glucose-1-phosphate to glucose-6-phosphate is taking place Since adrenaline stimulated the glycogenolysis in rat diaphragm it might have been expected that the quotient glucose-1-phosphate/glucose-1-phosphate + glucose-6 phosphate would increase further Instead this quotient decreased to 10 8 ± 0 9 per cent, i e it approached the equilibrium This reduction of 7 7 ± 1 2 per cent was significant ($P < 0\ 01$) This may mean that in rat diaphragm adrenaline enhanced the conversion of glucose-1-phosphate to glucose 6 phosphate, and also the phosphoglucomutase reaction Glucose-1-6-phosphate is considered to be a co factor to phosphoglucomutase (Najjar 1955), and it is not inconceivable that the production of this metabolite increased in parallel with glucose-1-phosphate and glucose 6-phosphate, and thereby hastened the glucose-1-phosphate ⟶ glucose-6-phosphate reaction Another *possible alternative which may be discussed is that glucose 6 phosphate was formed* from another metabolic than glucose-1 phosphate Glucose can be excluded however, since the experiments were performed in glucose-free solution

At equilibrium in the glucose 6 phosphate $\xrightarrow{\text{phosphoglucose isomerase}}$ fructose-6-phosphate reaction there is 68 per cent glucose-6-phosphate and 32 per cent fructose-6-phosphate (Slein 1955) In untreated rat diaphragm in our experiments this quotient was 68 32 After adrenaline the content of glucose-6 phosphate rose, so that the quotient became 82 18, *i e* the phosphoglucose isomerase reaction appeared as an enzymatic "bottle neck

The percentage increase of fructose 1 6-phosphate after adrenaline was greater, with statistical significance than the increase of fructose 6 phosphate, and it therefore seems probable that adrenaline stimulated the conversion of fructose 6-phosphate to fructose-1-6-phosphate A similar effect of adrenaline has been demonstrated

previously in vascular muscle (Beviz and Mohme-Lundholm 1964) It is of interest that Mansour (1963) showed that cyclic 3'—5' AMP stimulated the phosphofructokinase activity

The most pronounced effect of adrenaline in the carbohydrate metabolism of rat diaphragm was its stimulation of the glycogenolysis and increase of the content of glucose phosphates Since an increase by 15 per cent in the content of phosphorylase *a* has been shown in rat diaphragm in parallel experiments (Mohme Lundholm and Svedmyr 1964), it seems probable that this effect was quantitatively the most important However, adrenaline also appeared to stimulate the conversion of glucose-1 phosphate to glucose 6 phosphate, and fructose 6 phosphate to fructose 1 6 phosphate, and it is probable, therefore, that adrenaline has further points of attack in glycogenolytic chain apart from phosphorylase activation

Financial support for this investigation was provided by the Swedish State Medical Research Council and the Swedish National Association against Heart and Chest Diseases

References

BERGMEYER H U and H KLOTZSCH D-Glucose-1 phosphate In *Methoden der enzymatischen Analyse* (H U BERGMEYER ed) Verlag Chemie Weinheim 1962 131—133

BEVIZ A and E MOHME LUNDHOLM The effect of adrenaline on the hexosephosphate content of vascular smooth muscle *Acta physiol scand* 1964 62 109—114

BUCHER TH and H J HOHORST Dihydroxyaceton phosphate D Fructose 1 6-phosphate und D-Glycerinaldehyd 3 phosphate In *Methoden der enzymatischen Analyse* (H U BERGMEYER ed) Verlag Chemie Weinheim 1962 246—252

CORI C F and E W SUTHERLAND Effect of hyperglycaemic-glycogenolytic factor and epinephrine on liver phosphorylase *J biol Chem* 1951 *188* 531—543

FRIEDEMANN T E and J B GRAESER The determination of lactic acid *J biol Chem* 1933 *100* 291—308

HOHORST H J D-Glucose-6-phosphate und D Fructose-6-phosphate In *Methoden der enzymatischen Analyse* (H U BERGMEYER ed Verlag Chemie Weinheim 1962 a 134—138

KREBS H A and L KORNBERG A survey of the energy transformations in living matter *Ergebn Physiol* 1957 *47* 212

LUNDHOLM L and E MOHME LUNDHOLM The effect of adrenaline on the glycogen metabolism of smooth muscle *Acta physiol scand* 1956 *38* 237—254

LUNDHOLM L E MOHME LUNDHOLM and N SVEDMYR Comparative investigation of methods for determination of lactic acid in blood and in tissue extracts *Scand J clin Lab Invest* 1963 *15* 311 316

LYON J B and J PORTER The relation of phosphorylase to glycogenolysis in skeletal muscle and heart of mice *J biol Chem* 1963 *238* 1—11

MANSOUR T E Factors influencing activation of phosphofructokinase *Pharmacol Rev* 1966 *18* part 1 173 179

MOHME LUNDHOLM E and N SVEDMYR Influence of nethalide on the phosphorylase activating effects of adrenaline and isoprenaline in experiments on isolated rat diaphragm *Acta physiol scand* 1964 *61* 192 194

MCARDLE B Myopathy due to defect in muscle glycogen breakdown *Clin Sci* 1951 *10* 13—20

NAJJAR V A Phosphoglucomutase from muscle In Methods in Enzymology S P COLOWICK and N O KAPLAN eds Academic Press New York 1955 *1* 294—299

SCHMID R and R MAHLER Cronic progressive myopathy with myoglobinuria demonstration of a glycogenolytic defect in the muscle *J clin Invest* 1959 *38* 2044 2058

SLEIN M W Phosphoglucose Isomerase In Methods in Enzymology S P COLOWICK and N O KAPLAN eds Academic Press New York 1955 *1* 304—30[illegible]

SUTHERLAND E W The effect of the hyperglycaemic factor and epinephrine on enzyme systems of liver and muscle *Ann N Y Acad Sci* 1951 *54* 693

SUTHERLAND E W In Phosphorus Metabolism 2 577 eds McElroy W D and Glass, G Baltimore John Hopkins Press 1952

SUTHERLAND E W and T W RALL The relation of adenosine 3 5-phosphate and phosphorylase to the actions of catecholamines and other hormones *Pharmacol Rev* 1960 *12* 265—299

Acta physiol. scand. 1967. 69. 218—219

From the Metabolic Division, Department of Pharmacology, University of Gothenburg, Sweden

Influence of Adrenaline on the Content of Adenosine Triphosphate and Creatine Phosphate in Isolated Rat Diaphragm

By

Ella Mohme-Lundholm, Nils Svedmyr and Nandor Vamos

Received 21 October 1966

Abstract

Mohme-Lundholm, E., N. Svedmyr and N. Vamos *Influence of adrenaline on the content of adenosine triphosphate and creatine phosphate in isolated rat diaphragm* Acta physiol scand 1967 *69* 218—219

Adrenaline in a concentration of 10^{-6} g/ml increased the content of adenosine triphosphate (ATP) and creatine phosphate (CrP) in isolated rat diaphragm, by an average of 100 per cent 15 *min* after its addition

The effect of adrenaline on the carbohydrate metabolism has been studied in a previous investigation In addition to the well-known observation that adrenaline stimulates the glycogenolysis and lactic acid production in striated muscle we found that it also stimulated other processes in the Embden-Meyerhof glycolysis chain (Beviz *et al* 1967) The question of whether the stimulation of the carbohydrate metabolism by adrenaline also influences the content of high energy phosphate compounds (ATP + CrP) in skeletal muscle, does not appear to have been studied *Since under certain conditions adrenaline has been found to increase the content* of high energy phosphate compounds in smooth muscle (Lundholm and Mohme-Lundholm 1962, Beviz and Mohme-Lundholm 1965), it seemed of interest to study whether the same effect could be shown in striated muscle

Method

A rat was killed by a blow on the neck and its diaphragm divided into two parts Each part was then incubated for 30 min in 15 ml Krebs-Henseleit's bicarbonate buffer without glucose bubbled with 95 % O_2 in 5 % CO_2 Adrenaline in a concentration of 10^{-6} g ml was then added to one of the specimens, and after 15 min both pieces of the muscle were frozen with freon 12 at −80 C and after weighing were homogenized in 8 volumes 6 % perchloric acid After centrifugation the excess perchloric acid was neutralized immediately with potassium bicarbonate The extract was then analysed for ATP and CrP according to the method of Adam (1962

TABLE I The effect of adrenaline in a concentration of 10^{-6} g/ml on the contents of ATP and CrP in isolated rat diaphragm Mean of 8 tests P = probability that the effect was due to chance *Concentration in μmoles/g wet weight*

	ATP	CrP
Basal values	0 77 ± 0 08	1 44 ± 0 35
Increase over basal values after adrenaline	0 66 ± 0 18 P < 0 01	1 40 ± 0 54 P < 0 02

Results

Some of our previous experiments have indicated that the basal content of high energy phosphate compounds in the rat diaphragm are subjected to seasonal variations with a minimum during the month of August and a maximum during April The experiments described here were performed during August The mean content of ATP was 0 77 μmole/g muscle, and of CrP 1 44 μmole/g Adrenaline increased the ATP content in 6 out of 8 expts and the CrP content in 7 out of 8 expts, these contents being doubled, on the average (Table I)

Discussion

Adrenaline increased the contents of ATP and CrP in rat diaphragm in these experiments, and it seems probable that this effect was a result of the stimulation of the carbohydrate metabolism Supporting this assumption is the fact that when the phosphorylase-activating effect of adrenaline was inhibited in a medium rich in potassium, the elevating effect on the content of high energy phosphate compounds was also blocked (Lundholm *et al* 1967)

Financial support for this investigation was provided by the Swedish State Medical Research Council and the Swedish National Association against Heart and Chest Diseases

References

ADAM H Adenosine-5-triphosphate In Methoden der enzymatischen Analyse H U Bergmeyer ed Verlag Chemie Weinheim 1962 539—543

BEVIZ A and E MOHME-LUNDHOLM Influence of dihydroergotamine and adrenaline on the concentration of glucose 6-phosphate fructose 6-phosphate *adenosinetriphosphate and creatinephosphate* in bovine mesenteric artery *Acta physiol scand* 1965 65 289—291

BEVIZ A E MOHME-LUNDHOLM N SVEDMYR and N VAMOS The effect of adrenaline on the carbohydrate metabolism in striated muscle *Acta physiol scand* 1967 69 213—21

LUNDHOLM L and E MOHME-LUNDHOLM The effect of adrenaline and glucose on the content of high energy phosphate esters in substrate-depleted vascular smooth muscle *Acta physiol scand* 1962 56 130—133

LUNDHOLM L E MOHME-LUNDHOLM and N VAMOS Blockade of the phosphorylase activating effect of catecholamines in and cyclic AMP K depolarized isolated rat diaphragm *Acta physiol scand* 1967 *in press*

Acta physiol. scand. 1967. 69. 220—229

From the Department of Clinical Physiology, Glostrup Hospital, Copenhagen, Denmark

Renal Blood Flow, Circulation Times and Vascular Volume in Normal Man Measured by the Intraarterial Injection — External Counting Technique

By

JORGEN LADEFOGED and FRITZ PEDERSEN

Received 7 November 1966

Abstract

LADEFOGED, J and F PEDERSEN *Renal blood flow, circulation times and vascular volume in normal men measured by the intraarterial injection external counting technique* Acta physiol scand 1967 69 220—229

In six normal young men renal blood flow and renal mean circulation times for red cells and plasma were measured by external counting after injection into the renal artery of Xe 133 Cr 51 labelled red cells and I 131 labelled albumin Simultaneously the clearances of inulin and PAH and the blood pressure in aorta were measured Inulin clearance was in average 122 ml min 1 73 m² PAH clearance 663 ml min 1 73 m² renal cortical blood flow 5 4 ml g min mean renal blood flow 4 6 ml g min mean circulation time for erythrocytes 4 7 sec mean circulation time for plasma 5 5 sec and mean blood pressure in aorta 89 mm Hg The renal cortical vascular volume was 46 ml 100 g The data on renal blood flow obtained by the external counting technique correlated well with those obtained from the clearance measurements The mean circulation times were shorter than those measured by other investigators using indicator dilution techniques The cortical vascular volume which includes the blood in the renal veins and arteries, was greater than previously found in animal kidneys

In most investigations of the renal circulation in normal man the clearance technique has been used to measure renal blood flow and the dye methods to measure circulation times Recently measurements of the renal blood flow and circulation times for blood by external registration of the disappearance rates of radioactive tracers from the kidney after injection of the tracer into the renal artery have been introduced by Ladefoged and Kemp 1964 and by Pedersen and Baerenholdt 1966 The purpose of the present paper is to give data for the renal circulation in normal man as reference values for measurements in human kidneys under pathophysiological circumstances.

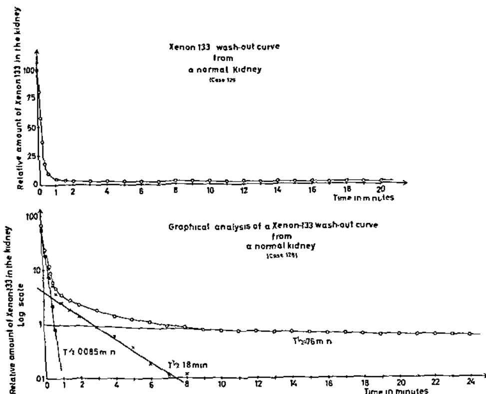


Fig 1 Xenon 133 desaturation curve from a normal kidney after injection of the gas dissolved in saline into the renal artery The upper part shows the curve in linear scale, the lower part the same curve in a semilogarithmic plot where three components are obtained by graphical analysis

Material and methods

a constant infusion of inulin (25 g per l) and PAH (4 g per l) in isotonic saline to keep the plasma concentrations on the desired levels The bladder was emptied by voluntary voiding The subjects

was used as the final clearance value

The mean blood pressure in aorta was recorded with a strain gauge manometer (Elema Schönander Type EMT 34)

The hematocrit was measured after spinning the specimen at a gravitational force of 1500 000 cm sec² for 30 min

From the PAH clearance the total renal blood flow (RBF_{PAH}) was calculated from

$$RBF_{PAH} = \frac{C_{PAH}/E_{PAH}}{1\text{-}Hct}$$

where E_{PAH} is the extraction ratio For all calculations the figure 0 9 was used according to the average value from 90 normal subjects (Smith 1951)

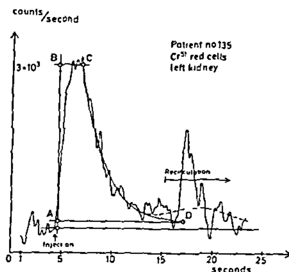


Fig 2 Registration curve from patient no 135 The curve was obtained by external counting after injection of Cr-51 labelled red cells into the left renal artery The area ABCD was used for the calculations The arrow to the left indicates the time of injection

The renal blood flow was also measured with the xenon-133 wash-out technique as described by Ladefoged (1966) Fig 1 shows a typical disappearance curve of xenon-133 from a kidney and its graphical analysis into three components From the half-time the clearance constants were obtained by $k = \ln 2/T1/2$ The clearance constants for each component were converted to blood flow values (F) by

$$F = \frac{k \cdot \lambda}{\varrho}$$

where λ denotes the partition coefficient for xenon-133 between kidney tissues and blood and ϱ the specific gravity of kidney tissue The value 0 7 (g ml) for the fraction λ/ϱ was used (Andersen and Ladefoged 1967) corrected for variations in the hematocrit by

$$\lambda Hct_x = 0.7 \frac{1.69}{1.05 - 0.013 \cdot Hct_x}$$

(Andersen and Ladefoged 1965)

The mean renal blood flow per gram ($mRBF_{Xe}$) was calculated from the initial slope of the disappearance curve of the xenon 133 as

$$mRBF_{Xe} = \frac{\ln 2}{T\frac{1}{2}} \cdot \frac{\lambda}{\varrho}$$

where T1 2 denotes the half-time of the slope line on semilogarithmic paper

The renal vascular resistance (R) was calculated from

$$R = \frac{MAP - MVP}{mRBF_{Xe}}$$

where MAP is the mean arterial pressure and MVP the mean pressure in the renal vein MVP was not measured For all calculations the normal value of 6 mm Hg was used (Munck 1958

Circulation times for red cells and plasma were obtained with the technique described by Pedersen and Baerenholdt (1966 Red cells labelled with Cr-51 (20—50 μC in 0 2—0 5 ml) were injected ... the renal artery by a double syringe permitting immediate washing out of the catheter ... l with I 131 (5 10 μC) was injected ... ion time was calculated according to ... ight Graphical analysis of the curves ... nd Baerenholdt (1966) ... t_0 The down-slope curve C-D was ... rculation The zero-slope curve was ... injection periode The area ABCD ... e calculation of $\bar{t}$ By this analysis a

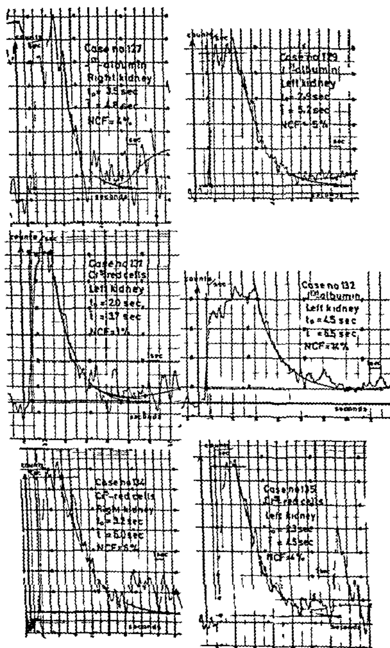


Fig 3 Original registration curves obtained by external counting after injection of I 131 albumin and Cr 51 labelled red cells in the renal artery The lines used for the graphical analysis are shown t_0 fastest circulation time $\bar{T}$ mean circulation time NCF non cortical fraction

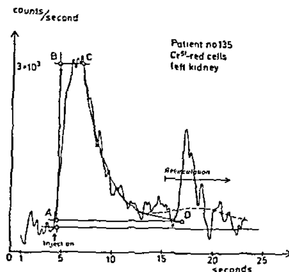


Fig 2 Registration curve from patient no 135 The curve was obtained by external counting after injection of Cr-51 labelled red cells into the left renal artery The area ABCD was used for the calculations The arrow to the left indicates the time of injection

values (F) by

$$F = \frac{k \lambda}{\varrho}$$

where λ denotes the partition coefficient for xenon-133 between kidney tissues and blood and ϱ *the specific gravity of kidney tissue The value 0 7 (g ml) for the fraction λ/ϱ was used* (Andersen and Ladefoged 1967 corrected for variations in the hematocrit by

$$\lambda Hct_x = 0\,7 \frac{1\,69}{1\,05 + 0\,013 \;\; Hct_x}$$

(Andersen and Ladefoged 1965)

The mean renal blood flow per gram ($mRBF_{Xe}$) was calculated from the initial slope of the *disappearance curve of the xenon 133* as

$$mRBF_{Xe} = \frac{\ln 2}{T\frac{1}{2}} \frac{\lambda}{\varrho}$$

where $T1/2$ denotes the half-time of the slope line on semilogarithmic paper

The renal vascular resistance R was calculated from

$$R = \frac{MAP - MVP}{mRBF_{Xe}}$$

where MAP is the mean arterial pressure and MVP the mean pressure in the renal vein MVP was not *measured* For all calculations the *normal* value of 6 mm Hg was used (Munck 1958)

Circulation times for red cells and plasma were obtained with the technique described by Pedersen and Baerenholdt (1966) Red cells labelled with Cr-51 (20—50 μC in 0 2—0.5 ml) were injected rapidly into the renal artery by a double syringe permitting immediate washing out of the catheter with saline 20 sec later 0 2—0 4 ml human albumin labelled with I-131 (5—10 μC) was injected Fig 2 shows a typical registration curve The mean circulation time was calculated according to

The area ABCD

By this analysis a

The reliability of the xenon 133 wash-out method for measurement of the cortical and mean renal blood flow has been demonstrated in animal experiments (Ladefoged *et al* 1965 Ladefoged 1966) The cortical blood flow calculated from the wash-out curves was in average 5 38 ml/g min which gives a ratio between the cortical and the mean renal blood flow of 0 12 The same ratio between cortical blood flow measured with the xenon 133 wash out technique and mean blood flow from flowmeter measurements was found in dogs by Ladefoged *et al* (1965) A ratio of 0 15 to 1 20 between the cortical blood flow from tissue desaturation curves of H_2 from the kindey and effective renal blood flow measured with the PAH clearance technique in dogs was found by Aukland Bower and Berliner (1964) From the blood flow rates in the dog kidney as calculated from dye dilution curves a ratio cortical blood flow/mean blood flow of 1 3 could be expected (Deetjen Brechtelsbauer and Kramer 1964) The extraction rate of PAH of 0 9 in normal man indicates a ratio of about 1 1 All these data agree as the small differences probably can be explained by difficulties in correct estimations of the kidney weights and by variations in the proportion of cortical to medullary tissue in the kidney

From the second component of the present xenon 133 wash out curves a blood flow rate of 0 50 ml/g min was calculated This figure is considerably lower than the figures for outer medullary blood flow known from the measurements of the dye transit times through outer medulla in dogs (Deetjen *et al* 1964) but it agrees fairly well with the desaturation rate of about 0 2 to 0 4 min^{-1} for hydrogen gas found by Aukland and Berliner (1964) in the outer medulla of the dog

Due to the counter current mechanism (Aukland and Berliner 1964) the trapping of xenon 133 in the fat capsule around the kidney and the recirculation (Ladefoged 1966) the third component is probably without any physiological significance

A renal weight of 276 g can be calculated from the total renal blood flow and the mean renal blood flow per gram This figure agrees well with the anatomical data of Greenwood and Brown (1913) and Roessle and Roulet (1932)

Fastest circulation time and mean circulation time The reliability of the external counting technique for measurements of the circulation times has been demonstrated in dog experiments by Pedersen *et al* (1965) It was found that the fastest circulation time measured by external counting was about 25 per cent too long The fastest circulation times in the present series were probably also too long due to a rather wide collimator field including the renal artery and renal vein

Only Reubi *et al* (1964) (Table III) have previously examined the fastest circulation time in normal man It varied from 1 5 to 4 5 sec which is close to the range reported here

The graphical analysis of the I 131 albumin and Cr 51 erythrocyte curves involves some problems as can be seen in Fig 3 By the graphical analysis a slowly passing fraction of the blood was excluded That this procedure some times is necessary appears from Fig 3 Case 13? In this case extrapolation to the background activity would have implied that t approached infinity The shape of the curve was

TABLE I Renal blood flow in normal man measured with the xenon-133 wash-out technique

Case no	Age years	Body surface m²	C_{IN} ml/min 1 73 m²	C_{PAH} ml/min 1 73 m²	F F	RBF_{PAH}
127	23	1 73	137	660	0 20	1222
129	23	1 96	109	565	0 19	1083
131	22	1 86	116	862	0 14	1544
132	26	2 02	123	550	0 22	1067
134	23	2 08	120	623	0 19	1211
135	27	1 88	124	710	0 17	1411
Mean		1 92	122	663	0 185	1257
S D		—	9	115	—	188

Six determinations of inulin clearance (C_{IN}), p amino hippuric acid clearance (C_{PAH}, filtration cortical blood flow per gram (cRBF$_{Xe}$), blood flow rates in components II (Comp II) and III component III (Comp III)

probably caused by a relatively high fraction of the injectate having been given into slowly perfused parts of the kidney

Experimental support of the reliability of the external counting technique is provided by the results of Pedersen *et al* (1965) In dog experiments the mean circulation time measured with the external counting technique was 18 per cent shorter than the time measured with a photocell installed on the renal vein This difference was explained by the fact that the latter time concerned all blood from the kidney, while according to the graphical analysis, the former only concerned *blood passing through cortex*

The mean circulation time for red cells or plasma in the normal human kidney has been determined by Shaldon, Silva, Lawson and Walker (1963) and Reubi *et al* (1964), with an indicator dilution method (Table III). The data of Shaldon et al concern blood passing through the whole kidney and their circulation times are therefore longer than ours, which only represent blood passing through cortex The time measured by Reubi et al is supposed to represent blood passing through cortex, but their figures are a little higher than ours This may be due to the lower renal blood flow in the subjects examined by Reubi *et al* (1964)

Cortical vascular volume Our measurements showed that the vascular volume in the kidney comprises about 46 per cent of the cortical weight Due to the counting geometry *this volume includes* the blood in the renal arteries and veins As the volume of these is approximately 5—15 ml the vascular volume without these vessels is about 30—40 ml per 100 g

The cortical vascular volume has not been measured previously in man In dogs Lilienfield, Rose and Lassen (1958 Emery *et al* 1959) and Deetjen *et al* (1964)

Blood flow rates ml/g min				Percentage of counts distributed initially		
$mRBF_{Xe}$	$cRBF_{Xe}$	Comp II	Comp III	Cortex	Comp II	Comp III
4 0	4 8	0 49	0 016	91	7	2
4 9	6 1	0 28	0 007	95	4	1
4 8	5 8	0 53	—	93	7	<1
3 8	4 4	0 63	0 017	92	6	2
4 1	4 7	0 56	0 015	87	11	2
5 7	6 5	0 52	0 018	94	4	2
4.55	5 38	0 50	0 015	92	6 5	1.5
0 72	0 86	0 12	0 004	2 7	2 6	2 4

fraction (F F) total renal blood flow (RBF_{PAH}) mean renal blood flow per gram ($mRBF_{Xe}$) renal (Comp III) and percentages of counts distributed initially to cortex component II (Comp II) and

TABLE II Circulation times and vascular volume in the normal human kidney measured by the external counting technique

Case no	Red cells t_0 sec	Red cells $\bar{T}$ sec	Red cells NCF per cent	Plasma t_0 sec	Plasma $\bar{T}$ sec	Plasma NCF per cent	Whole blood $\bar{T}$ sec	V ml 100 g	MAP mm Hg	R mmHg ml g min	$\frac{HCT_{kid}}{HCT_{LV}}$ per cent
12	3 9	5 4	0	3 5	4 8	4	5 1	41	100	24	107
129	2 1	3 4	0	2 9	3 2	3	4 4	43	[illegible]	14	69
131	2 0	3 7	1	2 1	3 9	4	3 8	37	86	1	95
132	3 1	5 3	3	4 5	[illegible]	14	6 0	44	90	[illegible]	86
134	3 2	6 0	6	4 8	[illegible]	6	6 4	50	91	21	93
135	2 3	4 5	4	3 1	6 0	5	5 3	57	91	15	81
Mean	2	4	2 3	3 5	5 5	[illegible] 3	5 2	46	89	18 8	88 5
SD	0 8	1 0	2 4	1 0	1 1	3 8	1 0	0	8 2	4 1	13 0

Six determinations of mean circulation time ($\bar{T}$) for red cells plasma and whole blood fastest circulation time (t_0) for red cells and plasma renal cortical vascular volume (V) non cortical fraction of red cells and plasma (NCF) mean arterial blood pressure (MAP) renal vascular resistance (R) and of dynamic hematocrit in the kidney cortex in per cent of the hematocrit in the large vessels (HCT_{kid}/HCT_{LV})

TABLE III. Collected data on renal circulation times, non cortical fraction of blood and intrarenal hematocrit in normal humans

Authors	Technique	Number of kidneys	$t_{0\ pl}$ sec	$\bar{t}_{rc}$ sec	$\bar{t}_{pl}$ sec	NCF per cent	cRBF ml/g min	$\frac{HCT_{kid}}{HCT_{Lt}}$ per cent
Shaldon *et al* (1963)	indicator dilution	5*	—	6.8	9.2	—	—	80 *
Reubi *et al* (1964)	indicator dilution	10	1.5—4.5	—	6—10	10	4.96*	
Ladefoged and Pedersen (1967)	external counting	6	2.1—4.8	3.4—6.0	3.9—6.7	5	5.38	89

$t_{0\ pl}$ = fastest circulation time for plasma $\bar{t}_{rc}$ = mean circulation time for red cells
$\bar{t}_{pl}$ = mean circulation time for plasma NCF = non cortical fraction of blood cRBF = renal cortical blood flow
$\frac{HCT_{kid}}{HCT_{Lt}}$ = dynamic hematocrit in the kidney cortex in per cent of hematocrit in the large vessels
*Controls, not defined Calculated from the data of Shaldon *et al* assuming a large vessel hematocrit of 45 per cent Calculated from the data of Reubi *et al* assuming that 90 per cent of the renal blood flow passes through a renal cortex weighing 105 g

measured a cortical vascular volume of 29, 33 and 19 ml per 100 g, respectively. The data were obtained from excised frozen dog kidneys. The difference between their results and ours can be explained by the inclusion of the blood in the vessels in our figures and probably by a decrease in the vascular volume in the dog kidneys due to anaestesia and manipulation (Burnett *et al* 1949, Habif *et al* 1951).

The total vascular volume in the normal human kidney has been found (Reubi *et al* 1964) to vary from 51 to 102 ml per kidney, which is consistent with a cortical vascular volume of about 40 ml per 100 g.

Intrarenal hematocrit. The ratio between the hematocrit in the kidney and in the great vessels was in average 0.89. From the data of Shaldon *et al* (1963) assuming an average hematocrit in the great vessels of 45 per cent, we have calculated an intrarenal hematocrit of 80 per cent of the hematocrit in the great vessels.

Non-cortical fraction of blood. The non-cortical fraction of blood was on the average composed of 2.3 per cent of the injected red cells and 6.3 per cent of the injected albumin. This fraction of the blood passes probably through medulla, capsule, pelvic, fat etc. and is of the same magnitude as that obtained in animal experiments with different methods (Kramer, Thurau and Deetjen (1960), Lilienfield, Maganzini and Bauer (1961), Swann and Nanninga (1962) and Ochwadt (1963)).

This work was supported by a grant from the Novo Foundation

References

ANDERSEN, A M and J LADEFOGED, Relationship between hematocrit and solubility of ^{133}Xe in blood *J Pharmaceut Sc* 1965, *54* 1684—85

ANDERSEN, A M and J LADEFOGED, Partition coefficients of xenon-133 between various tissues and blood in vivo *Scand J clin Lab Invest* 1967, *19* 000

AUKLAND, K, B F BOWER and R W BERLINER, Measurement of local blood flow with hydrogen gas *Circulat Res* 1964, *14* 164—187

AUKLAND, K and R W BERLINER, Renal medullary countercurrent system studied with hydrogen gas *Circulat Res* 1964, *15* 430—442

BOJESEN, E., A method for determination of inulin in plasma and urine *Acta med scand* Suppl. 1952, *266* 275—282

BRATTON, H E and E K MARSHALL, A new coupling component for sulfanilamide determinations *J biol Chem* 1939, *128* 537—550

BURNETT, C. H, E L BLOOMBERG, G SHORTZ, D W COMPTON and H K BEECHER, A comparison of the effects of ether and cyclopropane anaesthesia on the renal function of man *J Pharmacol exp Ther* 1949, *96* 380—387

DEETJEN P, H BRECHTELSBAUER and K KRAMER, Hämodynamik des Nierenmarks *Pflugers Arch ges Physiol* 1964, *279* 281—293

EMERY, E W, A H GOWENLOCK, A G RIDDELL and D A K BLACK, Intrarenal variations in hematocrit *Clin Sci* 1959, *18* 205—221

GREENWOOD, H and J W BROWN, A second study of the weight, variability and correlation of the human viscera *Biometrika*, 1913, *9* 473—485

HABIF, D V, E M PAPPER, H F FITZPATRICK, P LOWRANCE, C MCSMYTHE and S. E BRADLEY, The renal and hepatic blood flow, glomerular filtration rate, and urinary output of elektrolytes during cyclopropane, ether, and thiopental anesthesia, operation, and the immediate post operative period *Surgery*, 1951, *30* 241—255

KRAMER K, K THURAU and P DEETJEN, Haemodynamik des Nierenmarks, Capillaere Passagezeit, Blutvolumen, Durchblutung, Gewebshaematokrit und O_2-Verbrauch des Nierenmarks in Situ *Pflugers Arch ges Physiol* 1960, *270* 251—269

LADEFOGED, J and E KEMP, A new method for measurement of the divided renal blood flow in man by use of radioactive inert gas Proc II Int Congr Nephrol Prague 1963 *Excerpta Medica* International Congress Series no 78, 1964, pp 841—845

LADEFOGED, J, F PEDERSEN, U DOUTHEIL, P DEETJEN and E E SELKURT, Renal blood flow measured with xenon-133 wash-out technique and with an electromagnetic flowmeter *Pflugers Arch ges Physiol* 1965, *284* 195—200

LADEFOGED, J, Measurements of the renal blood flow in man with the xenon-133 wash-out technique Scand *J clin Lab Invest* 1966, *18* 279—315

LILIENFIELD, L S, J C ROSE and N A LASSEN, Diverse distribution of red cells and albumin in the dog kidney *Circulat Res* 1958 *6* 810—815

LILIENFIELD, L S, H C MAGANZINI and M H BAUER Blood flow in the renal medulla *Circulat Res* 1961, *9* 614—617

MUNCK, O, *Renal circulation in acute renal failure* Blackwell Oxford 1958 p 20

OCHWADT, B The measurement of intrarenal blood flow distribution by wash out technique Proc II Int Congr Nephrol Prague 1963 *Excerpta Medica* International Congress Series, no 78, 1964, p 62—64

PEDERSEN, F, J LADEFOGED, U DOUTHEIL and E E SELKURT Circulation times in the dog kidney measured by an external counting technique and by a dye dilution method *Pflugers Arch ges Physiol* 1965, *286* 36—43

PEDERSEN, F and O BAERENHOLDT Mean circulation time and fastest circulation time for red cells and plasma in the human kidney measured by an external counting method *Scand J clin Lab Invest* 1966 *18* 245—253

REUBI, F C., N GOSSWEILER, R GURTLER and A HUBER *The renal blood flow in acute renal failure Acute renal failure* Blackwell Oxford 1964 p 25—34

ROESSLE, R and F ROULET *Mass und Zahl in der Pathologie* Springer Verlag Berlin 1932, p 65—66

SHALDON S, H SILVA, T R LAWSON and J G WALKER Measurement of renal red cell and plasma transit times in acute renal failure *Proc Soc Biol N Y* 1963 a *11* 359—362

SMITH H W, The Kidney Oxford 1951 p 41—44 161 and 544 545

SWANN, H G and L B NANNINGA Proc XXII Int Congr of Physiol Sciences, Leiden 1962 *Excerpta Medica* International Congress Series Abstr no 236 237

THORBURN, G D, H H KOPALD J A HERD M HOLLENBERG C C C O'MORCHOE and A C. BARGER, Intrarenal distribution of nutrient blood flow determined with krypton 85 in the anestetized dog *Circulat Res* 1963 *13* 290 307

Acta physiol. scand. 1967, 69. 230—241

From the Department of Physiology, University of Turku, Finland

Skin D.C. Potentials and the Endosomatic Galvanic Skin Reaction in the Cat

By

A. H. Lang

Received 7 November 1966

Abstract

Lang, A. H. *Skin d.c. potentials and the endosomatic galvanic skin reaction in the cat.* Acta physiol. scand. 1967. *69*. 230—241.

D.c. potential recordings have been made on different skin areas in cats. A negative d.c. potential exists on the volar, hairless skin areas of the paws. This basal potential persists even though the limb is denervated, decreases reversibily when the arterial blood circulation is arrested for a short time in the limb, but disappears when the animal is sacrificed. A d.c. potential change is also induced by intravenously administered succinylcholine. It is not limited to the skin but seems to arise in the striated muscles. The endosomatic galvanic skin reaction, GSR, induced after a period of [illegible] both a [illegible] often simultaneously, but these two potential changes seem to be independent. The nature of the mechanisms maintaining and changing the basal potential and the relationships between the basal potential and the GSR are discussed.

The electrical behavior of the skin is a complex group of phenomena whose division into subgroups is usually done by applying methodical criteria. Many of these phenomena observed by different recording methods are generally believed to be due to membrane activity in different parts of the skin but the detailed mechanisms producing them and their mutual relationships have not been sufficiently clarified (for a general survey, see Rothman 1955.

The biological background of the so-called galvanic skin reaction, GSR, is probably best known. This reaction can be registered as a local potential change (endosomatic GSR) or a change in resistance or impedance (exosomatic GSR) and is usually thought to be related to sweat gland activity. The GSR's measured directly with a glass micropipette electrode located in the duct of a sweat gland of a cat are monophasic, negative potential waves of about 3 seconds duration when evoked by single shocks applied to the sympathetic trunk. Repetitive stimuli produce tetanic or fused responses (Shaver, Brusilow and Cooke 1962.

In studies using the GSR as an indicator of autonomic nervous activity, attention has up to the present been mainly directed to the variations in the amplitude of the reaction It has, however, been observed in this laboratory that at least in cats significant variations occur in the shape of the endosomatic GSR when stimulation sites and parameters and other experimental factors are changed For the purpose of quantifying these variations a special method has been developed (Lang 1967) which may give relevant results if the GSR is a product of only one bioelectric mechanism, whose mode of action can thus be expressed by the amplitude and time characteristics of the GSR

There are, however, indications that more than one potential mechanism may sometimes play a part in the production of the GSR, at least when the customary surface macroelectrode technique is used to record skin potentials It is a well-known fact that endosomatic GSR's of humans in response to, *e g*, sensory stimuli often include both a fast negative and a slow positive component (*cf*, *e g*, Holmquest and Edelberg 1964) Also in the GSR of a cat a slow, often positive component sometimes follows the faster negative potential phase Opinions vary about the origin of this slower component of the GSR From our own experience we know that also the shape of the fused GSR evoked in cats by efferent nerve tetanizations varies noticeably both in the same and different animals Some of these variations may be explained by assuming that a secondary mechanism causes distortions in the negative component of the GSR originating in the sweat glands

The purpose of the work described in this paper was to study d c potentials in the skin of the cat It was observed that a d c potential component of apparent biological origin exists in skin areas where the GSR is recorded *viz* the skin of the central and toe pads of the paws A second aim was to study the relations between the mechanisms causing changes in this skin d c potential and the GSR Only the phasic type of endosomatic GSR evoked by a *single* electric shock to the sudomotoric nerve fibres of a dissected sciatic nerve will be discussed below The problem of the relation between the skin d c potential changes and the variable shape of the fused GSR in response to *tetanic* stimulation will be the subject of a later study

Methods

The development of a reliable technique for recording the d c potentials and the GSR on cat skin has been in progress in this laboratory for over three years A number of electrode types ways of attaching them and the best location of the reference electrode have been tested The sympathetic trunk, the sciatic nerve or other peripheral nerves have been stimulated with electrical shocks to produce the GSR The results presented here are primarily based on systematic experiments with [illegible] cats in which skin potentials on isolated paw preparations were studied employing wet electrode skin contacts while the animals were restrained with succinylcholine

The sciatic nerve was chosen instead of the sympathetic trunk as the site of stimulation partly to avoid the possible complicating factors that could be due to changes in the synaptic transmission mechanism in the sympathetic ganglia The distal stump of the sectioned sciatic nerve can also be divided into two branches which both give a separate GSR on stimulation By this technique the [illegible] work in preparation The method thus required phar[illegible]

[illegible] anaesthetized intraperitoneally with Nembutal and [illegible]
[illegible] 45 mg of Nembutal and 100 mg of Urethane Som[illegible]

it was necessary to give a small additional dose during the course of a longer experiment A polythene catheter was inserted in the jugular vein for the infusion of succinylcholine after which artificial respiration was maintained The body temperature of the animal was followed and heat lamps were used to prevent hypothermia

A sciatic nerve was dissected, after which the paw was immersed in tap water for about half an hour, cleaned and dried One millilitre of Xylocaine Exadrine® (Astra) was injected into the tail at its midpoint and a tourniquet was applied at a point distal to the site of injection The soft tissues of the tail were removed to expose the tail cartilage to function as an inactive reference point isolated from the nervous system and the blood circulation

influence of the electrolyte on the electrical phenomena was to be expressly studied A layer of paraffin oil on the surface of the electrolyte prevented evaporation and consequent increase in concentration When potentials on the dorsal surface of the paw were recorded, a bored rubber stopper was fastened watertight with collodion to the shaved skin and the stopper hole was filled with the electrolyte

use of dry paste contact between the skin and the measuring electrode (zinc or silver) led to variations in the recorded d c potentials that were greater than those recorded with the electrodes just described

Grass model 5P1 F preamplifiers and a Grass model 5 six channel pen-recording polygraph were employed in the potential recordings The preamplifiers were in general d c coupled The *nominal input impedance was 200 kohm but the actual input impedance was 300—400 kohm* Even this latter impedance was too low in relation to the external resistance of the measuring circuit to permit direct quantitative measurements of the potential changes especially in the early stages of the experiments when the resistance of the skin was often of the same order as the input impedance of th

bringing th

amplifier

ion

sen

pli-

fier impedance in the same ratio as the recorded signal was divided A change in the source impedance then caused as great a relative change in the amplitude of the calibration signal as in the recorded biological signal The changes in the amplitude of successive calibration signals at the same time yielded a picture of the changes in the source impedance

The distal stump of the dissected sciatic nerve was stimulated bipolarly using silver silver chloride electrodes by square pulses single or repetitive, from a Grass model S 4 stimulator and a model SU — 4B isolation unit The stimulus intensity was controlled by an oscilloscope coupled parallel to the stimulating electrodes

The succinylcholine was injected intravenously by means of Palmer's Slow Injection Apparatus After an initial dose causing apnea and muscular inactivity, the rate of injection of succinylcholine was adjusted empirically to a certain optimum level which maintained a continued constant restraint without any greater changes in the d c potential in distal parts of the cat's paw see results, section 2, and discussion The injection rate varied from 15 to 10 mg of succinylcholine per kg per hr depending on the weight of the animal After the injection was terminated muscle activity returned within 10 20 min

pairs of series

e between

basal po-

nite proof

S test was

considered most ...

TABLE I Individual Basal Potential Values for Three Animals

Animal	Skin area on which recording was made	No of measurements (total number 100)	Basal potential Range	(mV) Mean	Statistical significance
Alive	Specific	45	− 4.6 — −37.0	−16.4	Yes
	Unspecific	22	+23.6 — − 7.0	+ 2.1	(P < 0.01)
Dead	Specific	18	+13.3 — − 3.9	+ 2.8	No
	Unspecific	15	+ 9.3 — − 7.1	+ 3.0	(P > 0.05)
Alive	Specific, intact limb	26	− 4.6 — −37.0	−14.5	No
	Specific, denervated limb	17	− 6.3 — −35.2	−19.5	(P > 0.05)

Results

1 *The basal potential* Considerable variations were observed in the basal potentials, *i e* in the d c potential differences between various skin areas and the employed reference area in anaesthetized animals The recorded basal potentials for different skin areas were, however, found to fall into two groups One group was formed by the values for ventral hairless skin areas of the paws including the central and toe pad regions, and the other group by the values for skin areas of the limbs and the body The former skin areas, which we call *specific*, were without exception negatively charged in relation to the reference area, whereas the basal potentials for the remaining or *unspecific* skin areas varied about zero The measured basal potentials for different skin areas in the same and different animals varied within wide limits, the basal potentials of the specific skin areas generally varied from 10—30 mV Such systematic differences in mean basal potential between specific and unspecific skin areas were not observed after the animal was sacrified In Table I the results of one hundred measurements on three cats are shown The recorded values are divided into three groups The first group consists of mean basal potentials for specific and unspecific skin areas of a living animal, the second group mean basal potentials for the same skin areas of a cat sacrified by asphyxiation, and the third group mean basal potentials for specific skin areas on intact and denervated limbs of a living animal On the basis of the statistical analysis, the difference between the basal potentials of the first group was significant, but the difference between the basal potentials of the second group was not significant, the values being practically equal The difference between the mean basal potentials of the third group was also not significant although the basal potentials of specific skin areas of the intact limbs were in some cats much lower than the basal potentials of areas on the denervated limbs when the spontaneous oscillations of the galvanic skin potentials revealed that the sudomotoric

activity in the intact limb was high This finding is in accord with the observation that sudomotoric activity induced by efferent nerve stimulation leads to a reduction of the basal potential (see section 3) By a reduction of the basal potential of a skin area we mean a decrease in the negativity

To establish the negativity of the basal potential of specific skin areas it is necessary that the electrolyte of the measuring electrode is in contact only with the hairless pad surface of a paw If the area of contact extends beyond this area, the measured basal potential is usually close to zero Both the nature of the electrolyte and its concentration influence the basal potential The presented values were measured employing a 1 N potassium chloride solution as electrode skin contact medium as it is known that potassium chloride in high concentrations eliminates diffusion type potentials of the skin at least in humans (Keller 1930 1931)

A correlation seemed to exist between the thickness of the epidermis and the basal potential of a specific skin area A thick dense epidermis usually gave a high basal potential value If the horny layer was rough became macerated and was removed on washing the basal potential was usually low When a small local lesion of the epidermis was produced which was not necessarily accompanied by bleeding the basal potential fell close to zero

When the basal potential was recorded *continuously* its level on an unspecific skin area if any potential difference in the beginning was recorded rapidly changed and approached close to zero The basal potential of a specific skin area, however changed only slightly if the sudomotoric activity in the measured area was nil owing to denervation of the limb or deep anaesthesia Usually both the basal potential and the skin resistance decreased very slowly This spontaneous decrease in the basal potential of a specific skin area did not exceed 2 5 mV during 10 min

2 *The d c potential changes after administration of succinylcholine* An intravenous dose of succinylcholine that effected complete paralysis of the striated muscles usually led to a marked change in the d c potential The magnitude shape polarity and duration of this change varied with the administered dose A biphasic potential wave which consisted of a slightly positive initial phase followed by a negative phase up to 20—30 mV in amplitude and 10—20 min in duration was often recorded This d c potential change caused by succinylcholine was not limited to the specific and unspecific skin areas but was recorded also from all subcutaneous tissues over the whole limb The shape of the potential change varied with the site where the measurement was made Potentials recorded from two points near each other e g a specific skin area and the subcutaneous tissue on the upper side of the same paw were close to each other in amplitude and shape The amplitude of the potential change was high for animals in which abundant muscle twitches occurred For the following reasons however, the potential change could not be considered to be an artefact resulting from movements of the limbs First a mechanically induced movement of the limb caused hardly any potential change with the employed range of amplification Second, the potential change recorded after a single dose of succinylcholine was

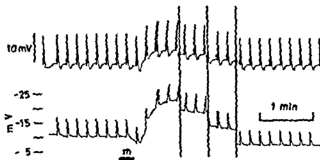


Fig 1 D c potentials produced by succinylcholine
Upper trace is a record from the right central pad of a hind leg of a cat taken with an RC coupled preamplifier (time constant 0 8 sec), the lower trace is a d c potential record Dissected sciatic nerves stimulated bilaterally with single supramaximal shocks at intervals of about 10 sec At "m" succinylcholine (1 mg/kg) was injected intravenously Sections extending over 1, 2 and 3 min were cut out from the continuous records at the vertical lines Note the constant amplitude of the GSR despite the considerable change in the d c potential level At the end of the d c potential change when the basal potential decreased to its minimum value, individual muscle twitches were observed as reactions to the electric shocks

always uniform and lasted up to 20 min and closely paralleled the extent and duration of muscle paralysis Thus, for example, the negative phase of the potential change rose to a maximum a few seconds after muscle relaxation began, and when the d c potential approached its original level after, say, 10 minutes, the muscle activity also returned

Succinylcholine caused no changes in the resistance of the specific skin areas or in the amplitude of the simultaneously measured GSRs, the phasic reactions were superimposed, as it were, upon the slowly varying d c potential level (Fig 1)

In view of these observations it seems obvious that the d c potential caused by succinylcholine has another origin than the negative basal potential whose existence is dependent on some kind of activity in the superficial layers of the specific skin areas (see discussion) But these two d c potentials and the changes they undergo cannot, of course, be distinguished when a d c recording is made only on a specific skin area For this reason it is necessary to administer succinylcholine by continued infusion after the initial dose to maintain a slight restraint while the basal potential is being recorded With the chosen rate of infusion (see methods) the change in the d c potential after succinylcholine infusion is started approaches its initial level within 10—20 min after which the d c potential change (recorded from subcutaneous tissue close to the specific skin area) does not exceed 0 6 mV during 10 min

3 *The potential changes after a period of rest*

The recording of the basal potential and the GSR was usually begun when 1 2 1 hr had elapsed from the dissection of the sciatic nerves The basal potential and the skin resistance were then usually high and the amplitude of the GSR following the first stimulation of the nerve stump low In addition the GSRs were often biphasic consisting of a fast negative and a slow mostly positive phase The latter component of the GSR may also be interpreted as a transient decline in the basal potential, especially as the basal potential often remained at a lower level after the positive phase than before the stimulation The duration of the positive phase was

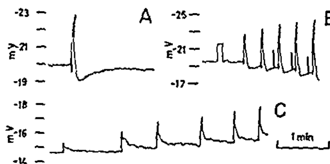
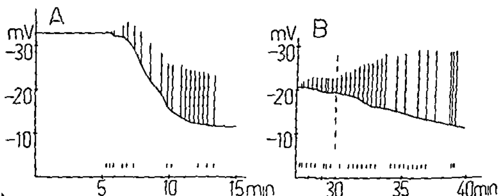


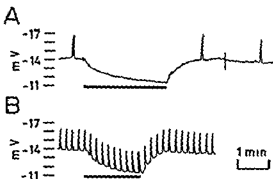
Fig 2 A, B, C *Effect of sciatic nerve stimulation on the GSR and the basal potential after a period of rest*
A GSR following a single supramaximal shock to the sciatic nerve After a slower (positive) phase, the basal potential level remains slightly lower than before the reaction
B Same preparation as in A, but repetitive shocks were now applied at intervals of 20 sec The amplitude of the negative phase increases at first without any change in the source impedance (no change in the amplitude of the four calibration signals, see methods) The total decrease of the basal potential during the stimulation period was 9 4 mV
C Record from another preparation taken as in B but the slow phase of the GSR is now negative in polarity and the basal potential increases (total change 1 5 mV) on continued activation



Figs 3 A and 3 B *Quantitative changes in the basal potential and the amplitude of the faster (negative) phase of the GSR after a period of rest*
The continuous line plots the basal potential and the lengths of the vertical lines represent amplitudes of the GSRs The lines immediately above the horizontal axis indicate the times of application of successive single stimuli to the sciatic nerve A and B show records for two different preparations Note change in time scale at the vertical broken line in B

30—50 sec (Fig 2 A) If successive single stimuli were applied at shorter intervals the positive phases of the GSR were superimposed and led to a stepwise decrease in the basal potential at the same time as a characteristic increase was observed in the amplitude of the negative phase of the GSR (Fig 2 B) After sufficient activation of the neuro-sudomotoric system, the basal potential finally decreased to a level that usually did not differ greatly from — 10 mV, the skin resistance decreased to a minimum value, and the amplitude of the negative phase of the GSR rose to a maximum value When the stimulation was discontinued for a longer period, the above sequence of changes was reversed, but the reversal was slow when a "wet" electrode skin contact was used

The initial potential changes just described did not, however, correlate exactly in magnitude or in time Figs 3 A and 3 B show the initial potential changes in two



Figs 4 A and 4 B Effect of occluding the regional blood circulation on the basal potential and the GSR

Two successive recordings from the central pad of the left hind leg of a cat In A only the changes in the basal potential were recorded except for the GSRs to sciatic nerve stimulation while the circulation was free and a third GSR after the femoral vein had been occluded (at vertical artefact) In B the artery was occluded while repetitive shocks were given The horizontal bars indicate the periods during which the femoral artery was clamped

animals In the former the change in the basal potential exceeded the change in the amplitude of the GSR whereas in the latter the situation was the reverse In both cases the increase in the GSR amplitude slightly preceded the change in the basal potential In some extreme cases the change of the basal potential was even of opposite polarity than in general, or no changes in the basal potential occurred despite a marked increase in the amplitude of the GSR (Fig 2 C) The reverse, a clear initial change in the basal potential without any increase in the amplitude of the GSR was never observed

Attempts were made to clarify the variations in the initial changes in respect of amplitude, time and polarity No clear systematic changes in the electrical behavior of the preparation after a period of rest were detected employing different electrolytes in different concentrations as electrode skin contact media

Figs 3 A and 3 B demonstrate also another fact which must be considered when GSR's are recorded to test the activity of the autonomic nervous system With a constant stimulus intensity (that can be supposed to activate a constant number of sudomotoric fibers) the recorded value of the GSR at a given moment is determined by the amount (that is by the mean frequency) of the preceding neurosudomotoric activity When, for example two identical neurosudomotoric effects are evoked at a short interval, the sudomotoric activity effected by the first stimulus has a potentiating effect on the second GSR which becomes evident as an increase in the amplitude The relative amplitudes of the GSR thus give an incorrect picture of the relative intensities of the central neural activities evoking the reactions In respect of the variations in amplitude of the GSR caused by this mechanism the scale is a sliding one in which only its lower end (relatively much preceding activity) is to some extent constant For drawing relevant quantitative conclusions from the amplitude of the negative phase of the GSR a constant level on this sliding scale is required It must be attained and maintained with a basal activation before and between the test stimuli In cases where the test stimulus is given frequently it itself serves as a sufficient "basal activation The "basal activation required varies from one animal to the next but is lower when a wet than when a dry electrode skin contact

ployed The requirement of a "basal activation" has been taken into consideration in this work

When the basal potential and the amplitude of the negative phase of the GSR had attained constant levels after sufficient 'basal activation" the slow component of the GSR sometimes remained, although its amplitude was only a fraction of the amplitude of the negative phase In many cases, however, it no longer existed

4 *The effects of arrested blood flow and asphyxia*

When the arterial blood circulation was arrested by clamping the femoral artery, a slow, reversible decrease occurred regularly in the basal potential of a specific skin area (Fig 4 A) but this decrease was in general smaller than that recorded when the animal died of asphyxia After the circulation was released the basal potential usually rose more rapidly to its original level than it decreased when the circulation was arrested Compression of the femoral vein led to no significant change in the basal potential A slight change in the basal potential was sometimes recorded on the intact dorsal skin of the paw after circulatory arrest, but this was much smaller than that recorded on a specific skin area The amplitude of the GSR did not change significantly during circulatory arrest of few minutes duration (Fig 4 B)

At the end of the experiments, the animals restrained with succinylcholine were caused to die of asphyxia by turning off the respirator The basal potential then rapidly decreased close to zero level The drop in the basal potential usually coincided with the onset of asphyxia revealed by changes in heart rate or other autonomic phenomena The amplitudes of the GSR's evoked by intermittent stimuli of constant intensity sometimes remained constant, but sometimes decreased The changes in the GSR and the basal potential were, however, usually asynchronous

Discussion

The d c potential phenomena of the skin *in vivo* have been studied to some extent in man Of these observations those of Keller (1930 1931) provide a starting point for the evaluation of the results of the later studies According to Keller essential requirements in these d c potential measurements are that the skin area under study *is not subjected to mechanical pressure and that diffusion* potentials and changes in skin temperature are eliminated. The significance of these factors has not always been realized in later investigations

Irrespective of the employed methods of investigation it has been repeatedly observed that an electrode applied to an intact skin surface is without exception negatively charged relative to a reference electrode on a damaged or drilled (Shachel 1959) skin area in man The negative potential difference is however, greatest when measured on palmar and plantar skin areas (Keller 1930, Floyd 1936) The fact that the skin areas of the palms in man, and according to our measurements the 'specific" skin areas in living cats are negatively charged relative to other skin

areas and not only relative to a damaged skin area shows that there prevails a d c potential source in the skin at least at the distal ends of the legs in man and cat

This potential difference may be explained by assuming that the skin in the distal parts of the leg with its thicker and denser structure offers a different passive resistance to diffusion than the epidermis elsewhere, but another possibility is that the d c potential is maintained by some active biological process We believe that our results support the latter alternative for the origin of the basal potential in the cat Firstly, even high potassium chloride concentrations do not eliminate the basal potentials of specific skin areas of the cat as would be expected if the ability of high potassium chloride concentrations to improve the rate of diffusion of cations through the human epidermis observed by Keller applied also to the cat epidermis Secondly, it is difficult to understand that occlusion of the local blood circulation (possibly because of the resulting hypoxia and/or hypothermia) causes a reversible, and fatal asphyxia an irreversible lowering of the basal potential if the difference in the basal potentials between specific and unspecific skin areas in living animals were due only to structural differences — One may ask what type of mechanism is responsible for a bioelectrical potential of this kind

Significant for the clarification of this question must be considered those investigations in which the skin has been stained with electrically charged dyes (Sulzenberger *et al* 1950) The findings suggest the presence of a negative charge at the terminal parts of the sweat ducts (the spiral ends and their orifices) This electrical charge tends to decrease on application of electropositive forces The view that the horny layer is a nonvital part of the epidermis that is unable to produce a constant electronegative charge is contradicted by histochemical observations which reveal that the keratinized cells which line the terminal parts of the sweat ducts exhibit a high enzyme activity (Montagna 1962)

We may assume that the electronegative basal potential recorded on specific skin areas of a living cat corresponds to the tonus potentials of Keller on palmar skin areas in man The low or nonexisting potential difference between unspecific skin areas and a reference point in the cat in relation to the negative tonus potential of Keller on proximal skin areas in man may be attributed to the presence of sweat glands only in the skin of the undersurfaces of a cat's paws The observations made seem to indicate further that the existence of the basal potential does not require intact innervation but is sensitive to disturbances in vital functions This would be readily understood if the basal potential is maintained by an energy consuming enzymatic mechanism

As far as the basal potential change after a period of rest is concerned it seems to be the result of the summation and fusion of the slow component of the GSR The fast negative component of the GSR has in general been attributed to sweat gland activity About the origin of the slow component in the cat two different opinions exist According to Lloyd 1961 both secretion and reabsorption of sweat take place in the sweat glands The input output relation of the sweat determines the state of filling of the sweat gland ducts upon which in turn the impedance of the skin depe-

sively depends As the time courses of the slow component of the GSR and the impedance change of the skin are the same, the former could be produced by cells effecting sweat reabsorption According to Shaver, Brusilow and Cooke (1962), however only the fast negative component of the GSR can be recorded with micropipette electrodes directly from the lumens of individual sweat gland ducts On the other hand, slow positive potentials can be recorded from epidermical cells when the sympathetic trunk is tetanized The method employed presupposes, however, that at least the horny layer of the epidermis is dissected away It is therefore not certain on the basis of the micropipette electrode recordings that the slow component of the GSR does not arise in the most distal, spiral end of the sweat duct One possible explanation for the mechanism that causes the slow component is that a decrease in skin impedance caused by or independent of sweat discharge loads the basal potential generator and thus causes a transient fall in the basal potential Also in this case the time course of the change in impedance and the change in basal potential should be identical The process may, however, be a more complex qualitative change in ion transport through cell membranes that maintains the basal potential This view would be in better accord with the observation that the slow component of the GSR and the change in basal potential are negative in polarity in rare cases The impedance and potential changes could be considered as different aspects of identical physiological processes as has been assumed to be the case in man (Wilcott 1964)

A common feature of these theories concerning the origin of the two components of the GSR is that they are produced by two, at least partly independent bioelectric mechanisms The data presented above seem to support this view The fast component of the GSR seems to be superimposed upon a fluctuating d c potential The physiological significance of the variations in the amplitude of the fast component will be the subject of a later report It has however now been demonstrated that the amplitude of the fast component of the GSR as a response to a stimulus of constant intensity to the efferent nerve changes as a function of prior sudomotoric activity

The d c potential recorded after administration of succinylcholine seems not to be related to the bioelectrical mechanisms of the skin discussed above The fact that this potential is correlated with the degree and duration of muscle paralysis caused by the pharmacological agent that it can be recorded also from subcutaneous tissue, and that it varies in magnitude and shape depending on the site of recording suggest that this potential arises in the muscles and spreads in a volume conductor The potential is in all probability due to a depolarizing effect around the end plate regions of the striated muscles caused by this kind of neuromuscular blocking agent (Burns and Paton 1951 Thesleff 1955) The basal potential of the skin can be measured without any significant error arising from the type of pharmacological restraint used when the method of succinylcholine administration employed in the present investigation is used

It would go too far to compare the views presented above with the proposed origins of skin potentials in man It may be noted however, that many observations point

to the existence of two mechanisms that give rise to the GSR in man (Darrow 1964 Edelberg and Wright 1964) A further problem is whether the d c potential levels in skin areas of distal parts of the limbs are directly regulated by the nervous system The d c potential level of the palmar skin area has, for example, been employed as a physiological criterion of higher neural functions (Venables 1963)

References

BURNS B D and W D M PATON Depolarization of the motor end plate by decamethonium and acetylcholine *J Physiol* (Lond) 1951 *115* 41—73

DARROW C W The rationale for treating the change in galvanic skin response as a change in

HOLMQUEST, D and R EDELBERG Problems in the analysis of the endosomatic galvanic skin response *Psychophysiology* 1964 *1* 48—54

KELLER P Die biologischen Grundlagen fur die elektrischen Potentiale der Haut *Arch Derm Syph* (Berl) 1930 *160* 136—139

KELLER P, Grund und Tonuspotentiale der Menschlicher Haut *Arc Derm Syph* (Berl) 1931 *162* 587—610

LANG A H A Mathematical definition of the tonicity of the galvanic skin reaction in the cat Acta physiol scand 1967 *69* In press

LLOYD D P C Action potential and secretory potential of sweat glands *Proc nat Acad Sci* (Wash) 1961 *47* 351—358

MONTAGNA W R E ELLIS and A F SILVER Eccrine sweat glands and eccrine sweating Oxford 1962 pp 6—29

ROTHMAN S Phys ology and Biochemistry of the Skin Chicago 1955 pp 9—25

SHACHEL B, Skin drilling A method of diminishing galvanic skin potentials *Amer J Psychol* 1959 *72* 114—121

SHAVER B A S W BRUSILOW and R COOKE Origin of the galvanic skin response *Proc Soc exp Biol* (N Y) 1962 *110* 559—564

SULZBERGER M B F HERRMANN R KELLER and B V PISKA Studies of sweating III Experimental factors influencing the function of the sweat ducts *J invest Derm* 1950 *14* 91—111

THESLEFF, S, The mode of neuromuscular block caused by acetylcholine nicotine decamethonium and succinylcholine *Acta physiol scand* 1955 *34* 218—231

VENABLES P H The relationship between level of skin potential and fusion of paired light flashes in schizophrenic and normal subjects *J Psychiat Res* 1963 *1* 279—287

WILCOTT R C The partial independence of skin potential and skin resistance from sweating *Psychophysiology* 1964 *1* 55—66

Acta physiol. scand. 1967. 69. 242—254

From the Nobel Institute for Neurophysiology, Karolinska Institutet, Stockholm 60, Sweden and the Department of Physiology, University of Umeå, Sweden

The Effect of Calcium on the Mechanical Response of Single Twitch Muscle Fibres of Xenopus Laevis

By

BERNHARD FRANKENHAEUSER and JAN LÄNNERGREN

Received 12 November 1966

Abstract

FRANKENHAEUSER, B and J LÄNNERGREN *The effect of calcium on the mechanical response of single twitch muscle fibres of Xenopus laevis* Acta physiol scand 1967 69 242—254

The effect of altered external calcium concentration on potassium induced contractures in single twitch muscle fibres of *Xenopus laevis* was studied It was found that an increase in $[Ca]_o$ shifted

high, both on twitch amplitude and contracture height the effects of a change in $[Ca]_o$ being seen in less than one second The results can be satisfactorily explained by assuming that the site of action for these calcium effects is the cell membrane

---

Depolarization of the excitable membrane of a muscle fibre beyond a critical level leads to contraction of the fibre This occurs independently of whether the depolarization is caused by an action potential, by an externally-applied current or by a depolarizing agent such as potassium chloride It is now commonly held that the membrane depolarization causes a release inside the muscle fibre of calcium which activates the contractile system (for reviews see Huxley 1964, Sandow 1965) This view is supported by the experimental finding that of all normally occurring ions, only calcium is effective in causing a local contraction when injected into muscle fibres or applied directly to isolated myofibrils (Heilbrunn and Wiercinski 1947, Niedergerke 1955, Podolsky and Hubert 1961, Caldwell and Walster 1963) The importance of calcium for the mechanical activity is further supported by the finding that calcium is necessary for the superprecipitation, or synaeresis, of actomyosin systems in the presence of ATP (Weber, Herz and Reiss 1964 *a*, *b*) However, calcium also has a specific effect on the excitable membrane as demon-

strated on nerve (Frankenhaeuser 1957, Frankenhaeuser and Hodgkin 1957) and on muscle (Ishiko and Sato 1957, Jenerick 1959, Luttgau 1963) Luttgau, for example, found in experiments with potassium contractures in single frog muscle fibres, that the curve relating peak tension to membrane potential as well as the restoration membrane potential curve was shifted towards more positive potential values when $[Ca]_o$ was increased. Luttgau also noted that the effect of altered $[Ca]_o$ occurred very rapidly, i e within a few seconds

The aim of the present investigation was to further examine the effect of altered $[Ca]_o$ on the mechanical response of muscle fibres Solutions of [Ca] above and below *normal were used and the effect on activation and inactivation was determined* The results from experiments with increased $[Ca]_o$ mainly confirm the findings of Luttgau (1963) It will also be shown that the effect of altered $[Ca]_o$ is very rapid both on contractures and on twitches

Specimens of *Xenopus laevis* were used, mainly because they are easy to maintain in good condition throughout the year The use of this species also permitted the selection, on the basis of optical properties, of fibres with similar contractile properties (Lannergren and Smith 1966) Single fibres were used to avoid long diffusion times

Methods

[illegible]

during the experiments as those of the transparent type

When a single fibre had been isolated it was mounted horizontally between two vertical glass rod [illegible]

per ods and between 15 and 25 cm sec during tests allo ing the contents of the channel to be replaced in less than 1/10 sec After mounting the fibre was allowed to rest for 30 60 m n Before the experiments were begun the fibre was extended to about 1 times its slack ength the sarcomere lengths were not measured At the end of the experiment the diameter was measured to the nearest 5 μm division in an eyepiece micrometer at several plac s along the fibre the mean was taken and rounded off to the nearest 5 μ n

Electrical stimulation of the fibre was br ught about by passing curr nt between two pla inum electrodes situated in the bottom of the r ght ha d part of the channel Pu ses of 1 msec duration and about 2 db above threshold were use l A ther set f electrodes the lef hand part of the channel was connected t the athode fo l er nput of a different al d an pl fier and wa used f [illegible]

[illegible] mM NaCl 11 KCl 2 [illegible] a usly bubbled w th a xture [illegible] tions with h gh [K] contained [illegible]

buffer was omitte l

All solutions w th hol ne Cl were made up fresh daily Glass-d st lled wa er was used through The experiments were carried out at room temperature 20—24 C

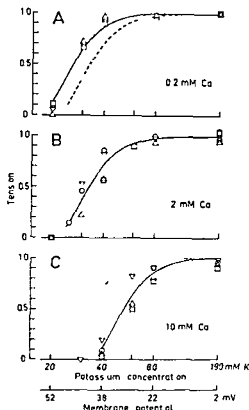


Fig 1 Relation between K concentration (logarithmic scale) and peak tension at three different $[Ca]_o$ A test was made in 190 mM K 2 mM Ca before and after each run in a certain $[Ca]_o$ and the mean of the tests was taken as unit tension for 1 *B* and *C*, respectively In *B* the amplitude of the first contracture and the control after a run in 2 mM Ca are given *A*, 0 2 mM Ca in equilibration (2 5 mM K) and test solutions (choline Cl replaced by [illegible] □, fibre 33, 100 μm, 3 2 kg/cm² The scale for membrane potential was obtained from microelectrode measurements on small bundles of fibres from the part of the muscle where the single fibres were dissected out

Results

When a muscle or a single muscle fibre is depolarized by the application of a solution with high potassium concentration it quickly contracts and then relaxes Hodgkin and Horowicz (1960) studied potassium contractures in single twitch fibres and described the curve relating peak tension of the contracture to log $[K]_o$ They found that tension began to rise at 20—30 mM-K and approached a maximum at about 80 mM-K

The first set of experiments in the present investigation was performed in order to see to what extent altered $[Ca]_o$ affected the relation between tension development and depolarization Ringer s solution flowed slowly around the fibre between tests This solution was changed to a modified Ringer's solution with the [Ca] to be used in the test and with NaCl replaced by choline Cl, the latter in order to avoid twitches when the high-K solution was applied Figure 1 *A*, *B* and *C* summarizes the results from contractures of four fibres obtained in 0 2, 2 and 10 mM-Ca, respectively The measurements were taken in the following way A run was first made in solutions with 2 mM-Ca, and after this, runs were made in the other calcium concentrations The maximum tension generally decreased somewhat during an experiment In order to check this running down of the preparation, tests were made with 190 mM-K, 2 mM-Ca before and after each run The results were evaluated only when

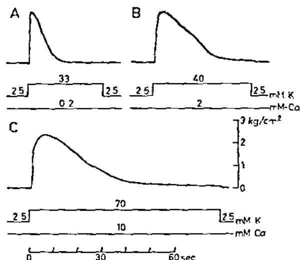


Fig 2 Tension records from one fibre (fibre 59, diameter 100 μm, maximal tension 2 6 kg/cm²) at three different $[Ca]_o$ The K concentrations were chosen to give submaximal contractures of equal amplitude in the various $[Ca]_o$ Note influence of $[Ca]_o$ on relaxation Records were taken in order *B*, *C*, *A*

the tension in the last test was within 80 % of the test before the first run The mean tension of the tests before and after a run was taken as unit tension in Fig 1 To facilitate a comparison between the results in the different calcium concentrations a smooth curve was drawn through the values obtained in 2 mM Ca (Fig 1 *B*) and the same curve was fitted to the values in *A* and *C* by shifting it along the horizontal axis A scale for the membrane potential at different potassium concentrations was obtained from measurements with micro electrodes on preparations of small bundles of muscle fibres at 2 5, 10, 20, 40, 80 and 190 mM-K and 2 mM Ca Measurements were also made at 40 mM-K in 0 2 and 10 mM-Ca There was no significant difference between the results in the three different calcium concentrations

The results presented in Fig 1 confirm the known dependence of peak tension on potassium concentration (Hodgkin and Horowicz 1960) and also confirm the finding that this dependence is changed by alterations of $[Ca]_o$ (Luttgau 1963) As can be seen from Fig 1, the effect of altered $[Ca]_o$ can be satisfactorily described as a mere horizontal shift of the tension log $[K]_o$ characteristic No certain indication was obtained that the curve was steeper in high $[Ca]_o$ as suggested in Fig 3 p 684) in the paper by Luttgau 1963 If the shift is expressed in mV a change from 2 mM to 10 mM Ca (five fold change was equivalent to a + 10 mV shift and a change from 2 mM to 0 2 mM Ca ten fold change) was equivalent to a shift of − 5 mV only The maximal tension in 190 mM K was slightly smaller in 10 mM Ca than in 2 mM Ca

The effect of altered $[Ca]_o$ on the time-course of the contracture

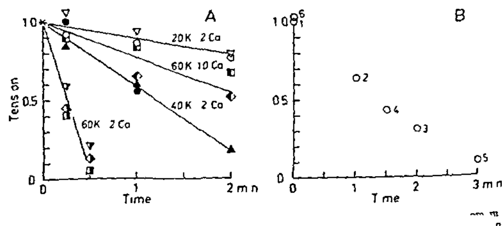
It is known that the time-course of a potassium contracture depends on the po[illegible] sium concentration which elicits the contracture Thus both the rate of ri[illegible] the rate of fall of tension increases with increased $[K]_o$ (Hodgkin and [illegible]

1960) The [Ca]$_o$ affects the time-course of the contractures caused by one and the same increase in [K]$_o$ (Luttgau 1963) These findings were verified in the present investigation An increase in [Ca]$_o$ especially decreased the rate of tension fall of the contractures

The effect of [Ca]$_o$ on the peak tension membrane potential curve could be satisfactorily described by a shift of the curve along the potential axis The following experiment was made in order to find out whether the relation between relaxation and membrane potential was shifted to the same extent by a change in [Ca]$_o$ as the tension membrane potential curve From the data in Fig 1 K concentrations were chosen which would give submaximal contractures of equal amplitude in 0.2 2 and 10 mM Ca respectively Figure 2 shows the resulting contractures when the chosen solutions were applied long enough for the fibre to contract and relax It is evident that although the peak tension in the three solutions was nearly equal the relaxation was much quicker in the solution with low [Ca] Thus the change in membrane potential required to balance the change in [Ca]$_o$ to give equal peak tension did not at all balance the calcium effect on the relaxation This point is further investigated in the experiments in the following section

The relation between long lasting depolarizations and the state of the contractile system

A solution with high potassium concentration (for example 190 mM K) causes a contracture of a muscle fibre the amplitude of which depends on the previous history of the fibre Preceding treatment of the fibre with conditioning solutions with increased K concentration thus reduces the height of the test contracture The degree of reduction is governed by the K concentration of the conditioning solution as well



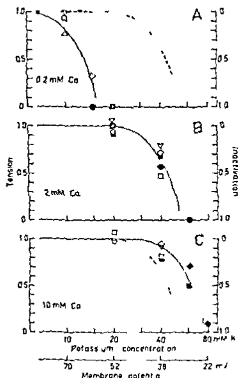


Fig 4 Relation between K-concentration (logarithmic scale) and inactivation at three dif...

...tion (right hand ordinate) is one minus tension in test contracture The scale for membrane potential is the same as in Fig 1 A, 0 2 mM Ca ...

... fibres ○, fibre 39, diameter 125 µm, maximal tension 2 3 kg/cm², □ fibre 40, 85 µm, 3 4 kg/cm², △, fibre 41, 85 µm 3 9 kg cm², ▽, fibre 42, 110 µm 4 0 kg/cm², ■ fibre 43, 95 µm 4 0 kg/cm², ○, fibre 44, 90 µm, 2 3 kg/cm², ◆ fibre 45, 110 µm, 2 7 kg/cm²

as the length of the conditioning period The term 'inactivation of the contractile system', or briefly 'inactivation', will be used in the following description of this effect of a conditioning treatment No implication is made here about which link, or links, are inactivated in the chain of events leading to tension production Inactivation is given as one minus the amplitude of the test contracture The size of the test contracture is measured in fractions of the response in the test solution without preceding conditioning Inactivation is thus zero in the resting fibre in normal Ringer's solution and unity in the completely refractory fibre Inactivation is possibly related to refractoriness and recovers after treatment with a high K solution (see Hodgkin and Horowicz 1960 Lüttgau 1963) but the relation is not simple

The aim of the experiments to be now described was to study the effect of changes in $[Ca]_o$ on the inactivation of the contractile system Preliminary experiments were performed on small bundles of muscle fibres from the iliofibularis muscle of *Bufo viridis* (2—8 fibres in each bundle) The preparation was soaked in a solution of increased [K] and a test with 190 mM K was applied after various periods of time (Fig 3 B) It is seen that the test response decreased with the duration of the conditioning period that the rate of change increased with increased $[K]_o$ and at any single $[K]_o$ was nearly constant Some measurements were made with 10 mM Ca in the conditioning solution As before the preparation was allowed to soak for about 2 min in 10 mM Ca Ringer's solution before the conditioning solution was applied Th

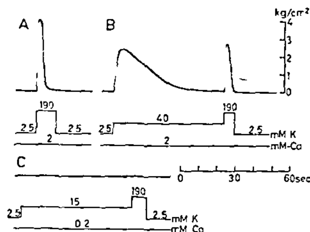


Fig. 5. Tension records from inactivation experiments on a single fibre. *A*, contracture in 190 mM K, 2 mM Ca without conditioning. *B*, conditioning for 1 min in 40 mM K, 2 mM Ca. Small inactivation (test with 190 mM K, 2 mM Ca) despite large contracture in first solution. *C*, conditioning for 1 min in 15 mM K, 0.2 mM Ca. No contracture in conditioning solution, complete inactivation. Irregularity of tension line just before the change to 190 mM K is a mechanical artifact. The records were taken in order *A*, *C*, *B*. Fibre 43, diameter 95 μm, maximal tension 4.0 kg/cm².

results show that the rate of inactivation was decreased by an increase in $[Ca]_o$ when [K] in the conditioning solution was constant. Figure 3 *B* shows results from a corresponding experiment in 2 mM-Ca on a single *Xenopus* fibre.

These experiments were not well tolerated by the fibres and complete measurements could not be carried out on one and the same preparation without a gradual decline in peak tension. Because of this, the following experiments, in which the effect of altered $[Ca]_o$ was studied in single *Xenopus* fibres, were made with a fixed conditioning time (1 min). Figure 4 gives the results of experiments on seven fibres that had been conditioned for 1 min in solutions, the composition of which are given the abscissa, before the application of the test solution (190 mM K, 2 mM Ca in all cases). The results clearly show that inactivation measured at the end of the 1 min period was affected by $[Ca]_o$. As in Fig. 1 a smooth curve was drawn through the values obtained in 2 mM Ca and this was fitted to the values from the other Ca concentrations by shifting it along the horizontal axis. The fit was satisfactory for the high $[Ca]_o$ values but not as good for the values obtained in low $[Ca]_o$ (Fig. 4 *A*). If the shift is expressed in mV, a change from 2 to 0.2 mM Ca was equivalent to −27 mV and a change from 2 to 10 mM Ca corresponded to +8 mV. The corresponding shifts of the activation curves were −5 and +10 mV, respectively (Fig. 1). From this it is apparent that high $[Ca]_o$ had comparable effects on activation and inactivation, whereas in low $[Ca]_o$ the effect on inactivation dominated.

Another aspect of this behaviour may be noted. The fibres were completely inactivated without ever having been activated in some of the low Ca solutions (Fig. 5 *C*), while in some normal Ca solutions they were nearly fully activated and very little inactivated (Fig. 5 *B*).

The effect of altered $[Ca]_o$ *on the recovery of twitches after treatment with high* $[K]_o$

A muscle fibre is refractory for some time after a high-K contracture of sufficient duration. During this refractory period neither twitch nor contracture can be elicited. The length of the refractory period depends on the K-concentration of the

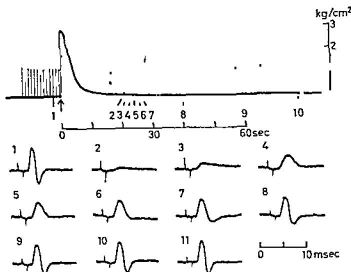
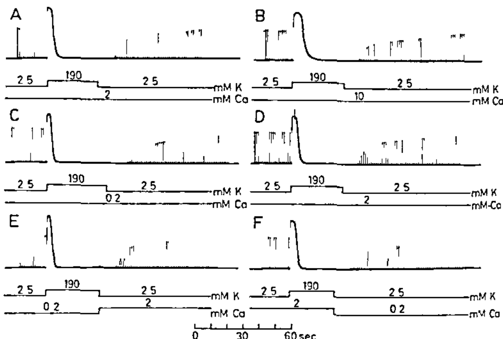


FIG 6 Simultaneous recording of tension (upper record) and action currents (lower records) in a single fibre Longitudinal current recorded with external platinum electrodes 4 mm apart Continuous stimulation of the fibre at 1/sec with shocks 2 db above threshold The contracture was induced with 190 mM K solution Arrows indicate when the high K solution was applied and when the change back to Ringer's solution was made, respectively Current records and corresponding twitches are indicated by figures Record 11 was taken after the fibre had been allowed to rest (unstimulated) for about 2 min Note decremental propagation in records 2—6 Fibre 49, diameter 95 μm, maximal tension 2 9 kg/cm²

solution causing the contracture (see Hodgkin and Horowicz 1960) This finding was confirmed here

Hodgkin and Horowicz suggested that the small twitch amplitude during the early phase of recovery was associated with decremental action potentials That this is indeed the case was demonstrated in experiments in which action currents were recorded with external electrodes simultaneously with the tension recording The action currents were clearly monophasic during the first phase of recovery and gradually returned to biphasic in about 20 sec (Fig 6)

The effect of changes in $[Ca]_o$ on the refractoriness was next studied Figure 7 shows records from one experiment in which the effect is demonstrated A single fibre was stimulated via external electrodes about once a second, tension was recorded and solution changes were made as indicated in the Figure The fibre was allowed to rest without stimulation in ordinary Ringer's solution for about 10 min between tests A modified Ringer's solution with the Ca concentration to be used in the contracture was applied 2 min before the contracture was elicited The contracture solution (190 mM-K) was applied for about 25 sec A comparison between experiments *A*, *B* and *C* shows that the duration of the refractoriness decreased with an increase in $[Ca]_o$ Record *D* is a control in normal $[Ca]_o$ In *E* the fibre was allowed to recover in 2 mM-Ca after a contracture in 0 2 mM-Ca and in *F* recovery took place in 0 2 mM Ca after a contracture in 2 mM-Ca A comparison of *E* and *F* with *A* and *C* indicates that the refractoriness depended not only on $[Ca]_o$ during the contracture but also to some extent on $[Ca]_o$ during the recovery. Even with altered $[Ca]_o$ both in the contracture and recovery solutions the change in the



contracture in 190 mM K 0 2 mM Ca *F* recovery in 2 5 mM K 0 2 mM Ca after a contracture in 190 mM K 2 mM Ca All records taken at the same ga n F bre 56 d ameter 105 μm peak tens on in the first contracture 2 6 kg cm²

absolute refractory time was small In 2 mM Ca this was 6 1 sec and it changed to 7 7 sec in 0 2 mM Ca and to 5 2 sec in 10 mM Ca respectively (means of three experiments individual values deviated up to 1 9 sec from the mean values)

The rate of action of changes in $[Ca]_o$

Some of the experiments described above indicated that the effect of a change of $[Ca]_o$ was quite rapid The following experiments give some information about how rapidly the effect occurs

It was clear (see Fig 1) that an increase to 35 mM K ought to be associated with quite different contractures in solutions with 0 2 and 10 mM Ca Figure 8 *A* and *B* shows two such contractures in one experiment The contracture in 0 2 mM Ca started rapidly was large and transient while the one in 10 mM Ca started slowly was much smaller and longer lasting In *C* $[Ca]_o$ was raised from 0 2 to 10 mM simultaneously with an increase in $[K]_o$ from 2 5 to 35 mM The peak tension was then only about half of that in *A* It is evident therefore that a large fraction of the effect of altered $[Ca]_o$ was rapid although not as rapid as the effect of the change in $[K]_o$ Distinct differences between the tension records in *A* and *C* are seen as early as 0 5 sec after the solution change The significance of the later part of the

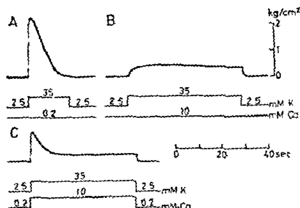


Fig 8 Contractures showing relative speed of action of change in $[K]_o$ and $[Ca]_o$. *A*, contracture in 35 mM K, 0·2 mM Ca *B*, contracture in 35 mM K, 10 mM-Ca *C* resulting contracture when $[K]_o$ and $[Ca]_o$ were changed simultaneously Note slowing of rising phase and reduction in peak tension compared to *A* Fibre 53, diameter 105 μm, maximal tension 3·4 kg cm²

A 2 10 2 mM-Ca

1 2 3 4 5 6 7 8

0 20 40sec

B 2 mM-Ca 10 mM-Ca kg/cm² 1 0

0 100 200 msec

C 1 2 3 4 5 6 7 8

tension records is difficult to judge, because the turning-off of the contraction controlling mechanism might be slow when return is made to a solution which itself causes a small contracture

In another type of experiment the effect was studied of altered $[Ca]_o$ on twitch amplitude and action current A single fibre was stimulated with current pulses which were about 2 db above threshold in 10 mM Ca Action currents were recorded with external electrodes Fig 9 shows the effect of a rapid change from normal Ringer's solution (2 mM Ca) to 10 mM Ca Ringer s solution while the fibre was stimulated once a second The amplitude of the first twitch after the solution change was somewhat diminished and then the twitch amplitude declined gradually to about 90 % of the original, this value being reached in 4—5 sec On return to 2 mM Ca the twitches recovered to the original amplitude in about the same time The current records (lower part of Fig 9) show that simultaneously with the reduction of twitch amplitude there was a decrease in propagation velocity and an increase in the duration of the action current When changes were made from 2 mM Ca to 0 2 mM Ca Ringer's solution, there followed a small increase in twitch amplitude and a decrease in action current duration

The observed changes in twitch amplitude were quite small This may be explained by two opposing effects of altered $[Ca]_o$ in these experiments In the case of increased $[Ca]_o$ for example there is a rapidly-occurring prolongation of the action potential as indicated by the early part of the current records which effect by itself is known to cause an increase in twitch amplitude (Edman and Grieve 1964, Sandow, Taylor and Preiser 1965) Thus the effect of the increase in threshold is counteracted That no transient increase in amplitude is seen after the solution change indicates that the effect on the threshold sets in as rapidly as that on the action potential

Discussion

In the present experiments it was found that a change in external calcium concentration was associated with a change in the depolarization required to activate the contractile mechanism A change in $[Ca]_o$ also markedly affected the membrane potential level at which the fibres became refractory or inactivated These effects are similar to those of calcium on the ionic permeabilities of the nerve membrane (Frankenhaeuser 1957 Frankenhaeuser and Hodgkin 1957) In both cases calcium acts by shifting the characteristics along the potential axis in the same direction The shifts obtained here for the activation curve were 5 mV for the change from 0 2 to 2 mM Ca and 10 mV for the change from 2 to 10 mM Ca, while the corresponding shifts for the inactivation curves were 27 and 8 mV, respectively

Lüttgau (1963 found a larger shift of the mechanical threshold when $[Ca]_o$ was increased than that described here (17 mV/e fold change compared to 5 mV in the present investigation) Luttgau also described a steepening of the tension membrane potential characteristic which was not seen in the present experiments The reason for this discrepancy is not clear One difference in the experiments was that Luttgau soaked the fibres about five times longer in the solution of increased

[Ca] than we did Adrian and Freygang (1962) found in increase in the mechanical threshold for local electrical stimulation of about 20 mV for a ten fold increase in $[Ca]_o$

Luttgau further found, for an e fold increase in $[Ca]_o$, a shift of 12 mV of the curve relating 'steady state inactivation' to membrane potential The corresponding value for the shift of the inactivation curve in the present experiments was 4 mV However, the two types of curve cannot be compared directly since they were obtained in different ways The values obtained by Luttgau represent the level to which the contractile system recovers during 1 min in a given $[K]_o$ after a contracture in high $[K]_o$, ours give the remaining available tension after 1 min at a corresponding $[K]_o$ If the two processes, inactivation and recovery, would reach a steady state in 1 min, then the 1 min values would measure the same change Clearly the inactivation process, tested as described here, never reached a steady-state The same might be true for the recovery process Hodgkin and Horowicz (1960) noted a slow decline' with time of the recovery Curtis (1964) in experiments on whole toe muscles of the frog found that the recovery process reached a maximum in about 1 min and then the response declined towards zero in 2—5 min In a control experiment we found some recovery in a solution (30 mM-K, 2 mM Ca), which, when applied directly, inactivated the fibre completely in about 4 min This indicates that recovery is not a simple removal of inactivation

A change in external [Ca] very rapidly affected the mechanical response of a fibre In the two types of experiment performed here, in which the rate of activation was tested, a clear effect could be observed within one second, and the full effect was seen in about 5 sec (Fig 8 and 9) In order to find out whether these findings are consistent with the idea that calcium exerts its effect on the membrane of the transverse tubules (T-tubules) of the sarcoplasmic reticulum, rather than at the surface membrane, an approximate calculation was made of the change with time of Ca concentration at a distance of 50 μm from the fibre surface It was assumed that Ca moved by free diffusion (Crank 1956 pp 11—14) The result of the calculation did not exclude the possibility that the site of action of calcium is in the tubules

As mentioned above the inactivation curve was shifted more than the activation curve in low $[Ca]_o$ This had the effect that in some solutions the fibres were fully inactivated without any preceding activation The finding is important because it clearly shows that the membrane, or the activating mechanism, can go directly from the resting state to the fully inactivated or refractory state without passing through the active state

It was also shown (Fig 5) that although a fibre had given a large contracture and subsequently relaxed (in 40 mM K 2 mM Ca) it was still but little refractory because 190 mM K gave a contracture which was about 70 % of the maximal This shows that there is no simple relation between inactivation and relaxation in high $[K]_o$.

An extrapolation of the effect of low $[Ca]_o$ to zero $[Ca]_o$ indicates that the fibres will be strongly inactivated at resting membrane potential, an idea already proposed

tension records is difficult to judge, because the turning off of the contraction controlling mechanism might be slow when return is made to a solution which itself causes a small contracture

In another type of experiment the effect was studied of altered $[Ca]_o$ on twitch amplitude and action current A single fibre was stimulated with current pulses which were about 2 db above threshold in 10 mM Ca Action currents were recorded with external electrodes Fig 9 shows the effect of a rapid change from normal Ringer's solution (2 mM Ca) to 10 mM Ca Ringer s solution while the fibre was stimulated once a second The amplitude of the first twitch after the solution change was somewhat diminished and then the twitch amplitude declined gradually to about 90 % of the original this value being reached in 4—5 sec On return to 2 mM Ca the twitches recovered to the original amplitude in about the same time The current records (lower part of Fig 9) show that simultaneously with the reduction of twitch amplitude there was a decrease in propagation velocity and an increase in the duration of the action current When changes were made from 2 mM Ca to 0 2 mM Ca Ringer s solution there followed a small increase in twitch amplitude and a decrease in action current duration

The observed changes in twitch amplitude were quite small This may be explained by two opposing effects of altered $[Ca]_o$ in these experiments In the case of *increased* $[Ca]_o$ for example there is a rapidly occurring prolongation of the action potential as indicated by the early part of the current records which effect by itself is known to cause an increase in twitch amplitude (Edman and Grieve 1964 Sandow Taylor and Preiser 1965) Thus the effect of the increase in threshold is counteracted That no transient increase in amplitude is seen after the solution change indicate that the effect on the threshold sets in as rapidly as that on the action potential

Discussion

In the present experiments it was found that a change in external calcium concentration was associated with a change in the depolarization required to activate the contractile mechanism A change in $[Ca]_o$ also markedly affected the membrane potential level at which the fibres became refractory or inactivated These effects are similar to those of calcium on the ionic permeabilities of the nerve membrane (Frankenhaeuser 1957 Frankenhaeuser and Hodgkin 1957) In both cases calcium acts by shifting the characteristics along the potential axis in the same direction The shifts obtained here for the activation curve were 5 mV for the change from 0 2 to 2 mM Ca and 10 mV for the change from 2 to 10 mM Ca while the corresponding shifts for the inactivation curves were 27 and 8 mV respectively

Luttgau (1963 found a larger shift of the mechanical threshold when $[Ca]_o$ was increased than that described here (17 mV/e fold change compared to 5 mV in the present investigation Luttgau also described a steepening of the tension membrane potential characteristic which was not seen in the present experiments The reason for this discrepancy is not clear One difference in the experiments was that Luttgau soaked the fibres about five times longer in the solution of increased

Acta physiol. scand 1967. 69. 255—256

From the Department of Anatomy, University of Helsinki, Finland

Effect of Hydrocortisone Administration In Utero on the Adrenaline and Noradrenaline Content of Extra-Adrenal Chromaffin Tissue in the Rat

By

OLAVI ERÄNKO, MATTI LEMPINEN and LIISA RÄISÄNEN

Previous studies have shown that hydrocortisone prevents the normal postnatal degeneration of the para aortic chromaffin bodies, so called organ of Zuckerkandl (OZ) (Lempinen 1964) Moreover, it causes the appearance of adrenaline in it, not normally present in the OZ of newborn rats (Eranko, Lempinen and Raisanen 1966) The present study was designed to find out whether administration of hydro cortisone during prenatal development of the OZ would have an effect on its catecholamine composition

Hydrocortisone was administered subcutaneously into the rat foetuses 16 days after copulation, i e , 5 days before the birth, by exposing the uterus of the pregnant rat under ether anesthesia and by injecting 1 mg of hydrocortisone (Hydro-Adreson, Organon) in each foetus Immediately after birth the foetuses were killed, and the retroperitoneal tissue block containing the OZ was examined chromatographically for catecholamines, as in the previous study (Eränkö *et al* 1966)

Fig. 1 shows that only the noradrenaline spot is visible in chromatograms obtained from OZ of normal newborn rats while both adrenaline and noradrenaline are present in the OZ of rats injected with hydrocortisone *in utero* Thus, hydrocortisone brought about the appearance of adrenaline in the OZ during a development period when this amine is never normally seen in it This observation strongly suggests that enclosure inside the cortical tissue in the adrenal gland significantly promotes the adrenaline synthesis of the chromaffin cells It fits admirably in with the recent observation (Axelrod 1966) that the level of phenylethanolamine N methyltransferase in the adrenal medulla is regulated by glucocorticoids secreted by the adrenal cortex



Fig 1 Fluorescence photograph of a chromatogram developed in phenol hydrochloric acid for 4 hrs Natural size

Left, adrenal cortical tissue, centre, adrenals of 4 normal newborn rats, right, adrenals of 2 newborn rats injected 5 days before birth with 1 mg of hydrocortisone The upper adrenaline spot is present in the last chromatogram only

References

AXELROD, J., Methylation reactions in the formation and metabolism of catecholamines and other biogenic amines *Pharmacol Rev* 1966 *18* 95—113

ERÄNKÖ, O, M LEMPINEN and L RÄISÄNEN, Adrenaline and noradrenaline in the Organ of Zuckerkandl and adrenals of newborn rats treated with hydrocortisone *Acta physiol scand* 1966 *66* 253—254

LEMPINEN, M, Extra adrenal chromaffin tissue of the rat and the effect of cortical hormones on it *Acta physiol scand* 1964 *62* Suppl 231

Acta physiol. scand. 1967. 69. 257—261

From the Department of Physiology, Karolinska Institutet, Stockholm, Sweden

Effect of Asphyxia on the Cortical Steady Potential in Adult and Fetal Sheep

By

E. EIDELBERG, G. M. KOLMODIN and B. A. MEYERSON

Received 14 October 1966

Abstract

EIDELBERG, E., G. M. KOLMODIN and B. A. MEYERSON. *Effect of asphyxia on the cortical steady potential in adult and fetal sheep.* Acta physiol. scand. 1967. 69. 257—261.

The [illegible]

The cortical steady potential (SP) of the adult mammalian brain has been considered to represent ionic gradients or polarization states in large groups of neurons. Slow changes in this DC potential have been found to be correlated to local as well as generalized cerebral activity (Goldring and O'Leary 1951, Libet and Gerhard 1962, O'Leary and Goldring 1964).

The development of the cortical steady potential has been studied during the postnatal period by Bures (1957, *cf.* also Laget and de Neverlee 1963) and during the prenatal period by Eidelberg *et al.* (1965). It was found that the SP is probably the first electrophysiological phenomenon to appear in the fetal sheep brain and is already present in the smallest fetuses that can be experimentally handled (about 1 g and 30 days gestation age).

It is well known that the immature nervous system has a greater ability to survive oxygen lack than the adult and it was therefore considered pertinent to perform a special study in the fetus of the effect of asphyxia on this early electrophysiological

Methods

The experimental techniques used have been described in detail in an earlier paper (Bernhard *et al* 1959) In short, pregnant ewes were anesthetized with a short acting barbiturate, tracheotomized and decerebrated They were usually immobilized with Flaxedil and maintained with artificial respiration The fetuses were delivered by Caesarean section and left connected to the placenta and uterus via the intact umbilical cord The fetal brain was exposed and the steady potential between

Results

The effect of asphyxiation on the SP of the *adult* brain has been analysed in many investigations and somewhat different results have been reported This is probably due to the techniques uesd for producing asphyxia as well as the method of recording (Bureš and Burešová 1957, Caspers *et al* 1963) Thus, clamping the carotid arteries results in a change of the "pia ventricular" SP (Goldring and O'Leary 1951) consisting of a postive shift during the first minute of asphyxiation followed by a significant negative shift of longer duration On the other hand, if the SP is recorded between the cortex and the bone of the skull and the same procedure of asphyxiation is used, the change consists of a marked and enduring negative shift without any previous positive shift (Leao 1947, v Harreveld and Stamm 1953)

The data obtained from an experiment on an adult sheep brain are shown in Fig 1 (filled circles) It appears that even after one minute of asphyxiation induced by stopping the artificial respiration the SP has become more negative and that this negativity rapidly increases to -10 mV after 4 min, at which time the respiratory pump was restarted (arrow) Following this procedure there was first a further increase of the negativity to 15 mV followed by rapid and complete restoration

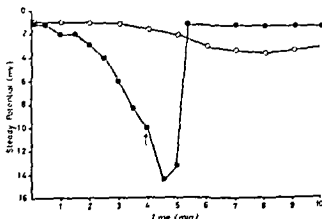


Fig. 1 *Steady potential shift induced by asphyxia*
Filled circles immobilized adult sheep artificial respiration stopped at zero time and restarted at arrow Open circles fetus 69 days (92 g) cord clamped at zero time

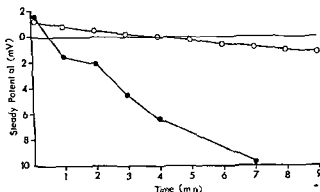


Fig 2 *Steady potential shift induced by asphyxia*
Filled circles mean values from 3 immobilized adult sheep, artificial respiration stopped at zero time Open circles *mean values from 9 fetuses of various ages 60—115 days, cord clamped at zero* time

In striking contrast to this marked shift in the adult brain are the results from asphyxiation of a fetus by clamping the umbilical cord during 10 min (Fig 1, open circles) There was no significant change during the first 3 min and during the following 7 min there was a very slow increase of negativity to 3 mV The difference in the rate of change of the SP during asphyxiation in the adult and fetal animal is further illustrated in Fig 2 which is based on mean values obtained from experiments on 3 adult *animals (filled circles)* and 9 sheep fetuses (open circles) of gestational ages varying between 60 and 115 days The ratio between the rate of fetal SP changes and that of the adult animal was 1 5 No relation was found between rate of SP change and fetal age within the gestational periods studied However, from the results obtained in a few preliminary experiments on fetuses close to full term it seemed as though in these cases the rate of SP change should amount to values between those for the whole group of younger fetuses and those of the adult animals If the maximal amplitude of the shift attainable in the fetus was about 3 mV, the difference in the rate of SP change observed during asphyxia, and consequently the difference in suspectibility to asphyxia could be suspected to be only apparent In some control experiments 1 M KCl was therefore applied to the cortex at the end of the asphyxiation period a further depolarization to between 5 and 10 mV was then obtained in the fetus (*cf* Eidelberg *et al* 1965), whereas in the adult no further significant shift was observed

Discussion

From experiments by Born *et al* (1956) using the same experimental species as in the present investigation data are available on the effect on blood oxygen saturation following suffocation of the ewe and clamping the cord of the fetus. It appears that as early as about 3 min after ventilation of the ewe with 10 % O_2, the arterial oxygen saturation of the maternal carotid blood was reduced to about 40 %

partial constriction of the umbilical vein reduced the oxygen saturation of the fetal carotid blood to about 27 % in 4 min. The rate of decrease of fetal oxygen saturation after clamping the umbilical cord will certainly not be slower than that achieved in the ewe by the method of suffocation The oxygen availability in the fetal cortex after clamping the umbilical cord measured by polarographic methods (Sundell and Theorell 1964) shows a considerable decrease after 3 min (*cf* also Misrahy *et al* 1960) It is thus not likely that different methods of producing asphyxia will account for the differences in the steady potential changes

The fetus as well as the newborn is known to be able to survive for longer periods of asphyxia than the adult Several studies suggest that this is due to a specific ability of the immature central nervous system to withstand asphyxia (see Libet *et al* 1941, Jílek *et al* 1961) This ability has been attributed to the fact that the fetal nervous tissue can utilize energy supplied by anaerobic processes, *e g* glycolysis (see e g Flexner *et al* 1956, Dawes *et al* 1963) The slow reaction of the fetal SP to asphyxia may be due to the fact that this potential during fetal life is maintained by anaerobic sources of energy and if this is so the SP or a part of the SP in the fetus would be a specific electrical correlate to these processes On the other hand, it cannot be excluded that the SP of the fetus and the adult is maintained by essentially the same metabolic processes but that the stores of anaerobically produced and utilized energy are larger in the fetal than in the adult brain (*cf* Palladin 1955)

During asphyxia there is aslo an increase of the pCO_2 and a decrease of the ph (*cf e g* Dawes *et al* 1963) but to what extent these changes are a consequence of or a necessary prerequisite for the fetal metabolic processes discussed above is not yet known.

It should aslo be pointed out that apart from differences in metabolic processes between fetal and adult animals there is also during the developmental period studied, a continuous and considerable structural change in terms of cortical lamination, dendritic growth and orientation of nerve cells In addition to this the development of the glial cell must be taken into account It has recently been shown that in adult amphibians Kuffler *et al* 1966 glial cells have large membrane potentials, about 90 mV and high internal K concentration These cells thus possess the same fundamental electrophysiological properties as nerve cells with respect to ability to serve as generators of DC potentials The rate of the development of these cells as compared to that of nerve cells is not known but it is possible that the mutual relation of these two types of cells is different in the fetus and in the adult It is thus possible that the SP in the adult brain is partly generated by other structures than in the fetus and this may be a factor that can explain the observed difference in susceptibility to asphyxia.

Regardless of which of these explanations is the correct one it is likely that the fetal SP represents mechanisms that are different from those generating neuronal and synaptic conduction manifested for example in the electrocorticogram which in the fetus as well as in the adult is extremely sensitive to asphyxia (Kolmodin and Meyerson 1966)

These investigations have been supported by grants from the Swedish Medical Research Council and Expressens Prenatalforskningsfond as well as, in the case of Dr Eidelberg (present address Barrow Neurological Institute, 350 W Thomas Road, Phoenix, Arizona), in part by a grant (GB 331) from the National Science Foundation (U S A)

References

BERNHARD, C. G , I H KAISER and G M KOLMODIN, On the development of cortical activity in fetal sheep *Acta physiol scand* 1959 *47* 333—349

BORN, G V R , G S DAWES and J C MOTT, Oxygen lack and autonomic nervous control of the fetal circulation in the lamb *J Physiol* (Lond) 1956 *134* 149—166

BUREŠ, J , The ontogenetic development of steady potential differences in the cerebral cortex in animals *Electroenceph clin Neurophysiol* 1957 *9* 121—130

BUREŠ J and O BUREŠOVÁ, Die anoxische Terminaldepolarisation als Indicator der Vulnerabilität der Grosshirnrinde bei Anoxie und Ischämie *Pflügers Arch ges Physiol* 1957 *264* 325—334

CASPERS, H , E SCHUTZ and E J SPECKMANN, Gleichspannungsveränderungen an der Hirnrinde bei Sauerstoffmangel *Z biol* 1963 *114* 112—126

DAWES, G S , J C MOTT H J SHELLEY and A STAFFORD, The prolongation of survival time in asphyxiated immature foetal lambs *J Physiol* (Lond) 1963 *168* 43—64

EIDELBERG, E , G M KOLMODIN and B A MEYERSON Ontogenesis of steady potential and direct cortical response in fetal sheep brain *Exp Neurol* 1965 *12* 198—214

FLEXNER, L B , J B FLEXNER and L HELLERMAN, Biochemical and physiological differentiation during morphogenesis *J cell comp Physiol* 1956 *47* 467—482

GOLDRING, S and J L O LEARY, Experimentally derived correlates between EEG and steady cortical potential *J Neurophysiol* 1951 *14* 276—288

VAN HARREVELD, A and J S STAMM Cerebral asphyxiation and spreading cortical depression *Amer J Physiol* 1953 *173* 171—175

JILEK, L , J MOUREK, S TROJAN, E TRAVNIČKOVA, L KRULICH and J FISCHER On the question of development of phenomena of adaptation in CNS during ontogenesis *Plzeň lék Sbor* 1961 Suppl 3 103—112

KOLMODIN, G M and B A MEYERSON 1966 *In preparation*

KUFFLER, S W , J G NICHOLLS and R K ORKAND Physiological properties of glial cells in the central nervous system of amphibia *J Neurophysiol* 1966 *29* 768—787

LAGET, P and H DE NEVERLÉE Etude du potentiel stable et de la depression envahissante chez le lapin en cours de développement *J Physiol Paris* 1963 *55* 278—279

LEAO, A A P , Further observations on the spreading depression of activity in the cerebral cortex *J Neurophysiol* 1947 *10* 409—414

LIBET, B , J F FAZEKAS and H E HIMWICH The electrical response of the kitten and the adult cat brain to cerebral anemia and analeptics *Amer J Physiol* 1941 *132* 232—238

LIBET, B and R W GERHARD An analysis of some correlates of steady potentials in mammalian cerebral cortex *Electroenceph clin Neurophysiol* 1962 *14* 445—452

MISRAHY G A , A V BERAN J F SPRADLEY and V P GARWOOD Fetal brain oxygen *Amer J Physiol* 1960 *199* 959—964

O LEARY, J L and S GOLDRING D-C potentials of the brain *Physiol Rev* 1964 *44* 91—125

PALLADIN A V *Metabolism of nucleic acids in the brain during its development* In H Waelsch Ed ; Biochemistry of the developing nervous system Academic Press New York 1955

SUNDELL, H and K THEORELL 1964 Personal communication

Acta physiol. scand. 1967. 69. 262—269

From the Physiological Institute and the Broegelmann Research Laboratory for Microbiology
University of Bergen, Norway

Serum Proteins in the Hedgehog

By

BODIL LARSEN[1] and OLAV TONDER

Received 18 October 1966

Abstract

LARSEN, B and O TONDER *Serum proteins in the hedgehog* Acta physiol scand 1967 *69* 262—269

Hedgehog serum proteins were studied by physical, chemical and immunological methods The paper electrophoretic and immunoelectrophoretic patterns did not differ greatly from those of human serum In paper electrophoresis two beta-bands were found, the $beta_2$-band being the most conspicuous In the immunoelectrophoretic pattern, the form of the "rapid" lipoprotein line was found to vary with temperature and duration of storage of serum Autoradiography showed transferrin in hedgehog serum to be a $beta_1$-globulin Antibody activity was found in two different fractions after gel filtration or ultracentrifugation of serum, probably representing antibodies of the 19S and 7S types

Most of the year the hedgehog is homoiothermic with a body temperature of 34° C but during hibernation in winter it becomes poikilothermic with a body temperature only slightly above the temperature of the surroundings This animal is therefore well suited for studies on the influence of body temperature on various biological processes

The object of the present study was to determine the effect of hypothermia and hibernation on the response to artificial immunization For this purpose it was necessary to become familiar with the serum proteins of the non-hibernating hedgehog Few reports have appeared in the literature concerning the serum proteins of this animal Biorck *et al* (1956) studied the seasonal variations of hedgehog plasma proteins and lipids by paper electrophoresis, and Suomalainen and Karppanen (1956) also studied the variation in concentration of albumin and globulins in plasma Both studies revealed a relative decrease of globulins during hibernation Physico-chemical or immuno-chemical characterization of the proteins has so far not been attempted

In the present work, hedgehog serum was fractionated by various procedures, and the proteins were characterized and identified by physical, chemical and immunological techniques

[1] Fellow of the Norwegian Research Council for Science and the Humanities

Materials and methods

Antiserum to hedgehog serum was obtained from a rabbit immunized by intracutaneous and subcutaneous injections once a month of 1 ml of neat hedgehog serum in Freund adjuvant, according to the procedure outlined in Kabat and Mayer (1961). The rabbit was bled by cardiac puncture three weeks after the third injection.

All sera were stored frozen at – 20 °C, usually in portions of 1 ml.

Red cells. Animal red cells were obtained from whole blood collected in Alsever solution. Before use, the red cells were washed four times in 10 volumes of phosphate buffered saline, pH 7.2. They were finally packed at 1000 g for 10 min.

Agglutination test. The sera were titrated by twofold dilutions in 0.1 ml volumes of buffered saline. To each tube was added 0.1 ml of a one per cent suspension of red cells. The racks were left at 4 °C for at least 3 hrs. The agglutination was recorded after the tubes had been centrifuged for 30 sec at 1000 g.

Paper electrophoresis. Electrophoresis of serum or serum fractions was carried out at room temperature using a 0.1 M veronal buffer pH 8.6 and a cell of the horizontal strip type accommodating 8 strips. Electrophoretic separation was carried out for 16 hrs at 8 mA and 60 V, the voltage gradient being 2.6 V cm⁻¹.

Proteins were stained with amido black or bromphenol blue. Lipoproteins were stained with Sudan black or oil red O and haptoglobins with o-dianisidine (see Ivor Smith 1960).

The proteins of the strips were evaluated by reflection densitometry in an automatic recording and integrating densitometer (Shandon, London).

azide

Immunoelectrophoresis. The micromodification of immunoelectrophoresis was employed using com-
constant voltage of 250 V was
e serum proteins. The antisera
The slides were stained with
ography using ⁵⁹Fe as ferrous
chloride was employed for identification of transferrin. The radioactivity of the line was recorded on a Kodak X-ray film DF 45.

Preparative ultracentrifugation. Serum was fractionated in a 10 to 40 % sucrose gradient by ultracentrifugation for 16 hrs (Fudenberg and Kunkel 1959). After centrifugation a hole was punched in the bottom of the tube and the [illegible]

used without being dialyzed.

Experiments and results

Paper electrophoresis

For protein separation 5 µl of undiluted serum were applied to each paper. Sera from 13 hedgehogs were analyzed, the blood samples being taken prior to immuniza-

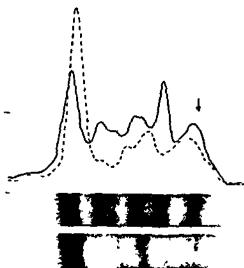


Fig 1 Paper electrophoresis of hedgehog and human sera The strips were stained with amido black Upper strip pooled hedgehog serum Lower strip pooled human serum The diagrams show the results after scanning of the strips Continuous line pooled hedgehog serum Broken line pooled human serum Arrow indicates place of application of samples Negative electrode to the right

TABLE I Total serum protein (micro-Kjeldahl) in hedgehog serum, and the relative values of serum proteins determined by paper electrophoresis Mean values and standard deviations for 13 non hibernating hedgehogs

Total serum protein g/100 ml	Serum fractions in per cent					
	albumin	$alpha_1$	$alpha_2$	$beta_1$	$beta_2$	gamma
7 0±0 88	27±3 7	12±2 1	8±1 6	15±1 6	18±1 6	20±3 6

tion After staining with bromphenol blue, 6 bands could always be distinguished Fig 1 shows the pattern obtained with pooled hedgehog serum For comparison, pooled human serum was run simultaneously

The average amount of total serum protein and the average relative amounts of protein in each electrophoretic band are shown in Table I

Prealbumin did not give a distinct band, but a small shoulder of prealbumin near the base of the albumin peak was included in the albumin fraction When electrophoresis was performed at 4° C this shoulder became more distinct The gamma-peak often had one or two shoulders, probably representing two or three proteins with different mobilities

There was always good separation between the bands except for the $alpha_2$ band which, when scanned, often appeared only as a shoulder on the $alpha_1$-peak and not as a separate peak

For lipoprotein staining, 50 μl of serum were applied to each strip before electrophoresis Sudan black or oil red O gave a diffuse band extending from the $alpha_1$ — into the albumin region No beta lipoprotein band was found

For haptoglobin staining, 10 μl of serum were applied to each strip before electrophoresis Staining with o dianisidine gave a $beta_1$-band and often a very weak band

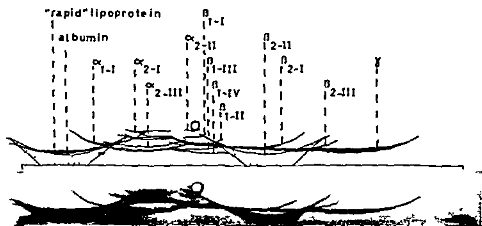


Fig 2 Immunoelectrophoretic pattern of hedgehog serum Hedgehog serum was separated by electrophoresis at 18 C The slide was stained with amido black Identity of the lines is indicated in the drawing Negative electrode to the right

between the alpha$_2$ and the beta$_1$ band Serum to which haemoglobin had been added prior to electrophoresis gave similar bands When excessive amounts of haemoglobin were added, a zone corresponding to free haemoglobin appeared in the gamma region

A mobility constant was not calculated for hedgehog haemoglobin but, when run simultaneously with haemoglobins from sheep, rabbit, mouse and guinea pig, hedgehog haemoglobin moved the least, moving only slightly towards the anode

Immunoelectrophoresis

Using rabbit anti hedgehog serum in the antibody trough immunoelectrophoresis of hedgehog serum gave 14 lines (Fig 2) Only eight of these lines were distinct in all sera tested

Albumin region The first line was found to represent 'rapid lipoprotein It was rather a long line, only slightly curved, which began in the prealbumin region and passed into the alpha$_1$ region The form of this line showed temperature dependence When electrophoresis was performed at room temperature or at 37° C, the line was longer and consisted of two curves, one in the albumin region and one in the alpha$_1$-region. This temperature dependence is probably a function of the duration of storage of serum When serum from freshly drawn blood was used (July) immuno electrophoresis at room temperature gave only a single curve as at 4° C After one week of storage (frozen) the serum still gave only one curve at room temperature Serum from the same hedgehog drawn one month earlier and stored frozen, gave a line with two curves (Fig 3) The serum had been thawed and frozen several times

Immunoelectrophoretic analysis of fractions obtained after gel filtration of whole serum showed the "rapid' lipoprotein to be in the second peak of high protein

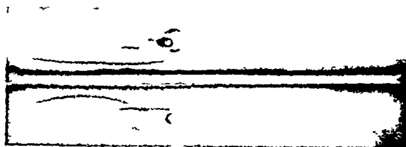


Fig 3 Immunoelectrophoretic pattern of hedgehog serum Hedgehog serum was separated by electrophoresis at 18 C. The slide was stained with oil red O to show lipoproteins Upper half hedgehog serum stored for two month Lower half fresh hedgehog serum Negative electrode to the right

concentration Like serum from most other mammals, hedgehog serum gives three peaks of high protein concentration when fractionated on Sephadex G 200 Ultra centrifugation of hedgehog serum left the rapid lipoprotein in the fraction which contained most of the albumin and in the fraction prior to this one Staining a simple agar electrophoretogram of hedgehog serum with oil red O showed a wide lipoprotein band extending from the alpha, to the prealbumin region with a more intensely stained band in the alpha region The alpha, lipoprotein band represents slow lipoprotein and will be discussed later Counter staining with amido black clearly showed the wide lipoprotein band to extend farther towards the anode than the albumin band

The second line was that of albumin It was a line of heavy precipitation and always net After gel filtration of hedgehog serum albumin was found in the third peak of high protein concentration

Alpha₁ region This region contained only one line It was a distinct strongly curved line the center of the curve being slightly displaced towards the anode This protein was found in the first peak of high protein concentration after gel filtration and in the bottom fractions after ultracentrifugation of hedehog serum It was destroyed by mercaptoethanol and behaved as an alpha, globulin in paper electrophoresis Accordingly it must be an alpha, macroglobulin

Alpha region In the alpha, region there were several lines the most prominent being that part of the prealbumin lipoprotein line extending into the alpha, region when electrophoresis was performed at room temperature The alpha 1 protein was also a lipoprotein It gave an almost straight line the protein diffusing only slightly towards the antibody trough This line represented slow lipoprotein At times a short, curved lipoprotein line was superimposed on the anodic part of the alpha -1 line The "slow lipoprotein was found in the fractions of the first peak of high protein concentration after gel filtration This protein was also destroyed by mercaptoethanol

The alpha II line was short and less anodic than the alpha I forming a curve directly below the antigen well and continuing as a straight line towards the anode This line was not distinct in all sera tested

Immediately above the alpha part of the prealbumin line there was a thin line, alpha$_2$ III. This was often difficult to observe and might also appear between the albumin and the prealbumin lines. The protein constituting this line was found in the fractions containing albumin after gel filtration or ultracentrifugation of serum.

Beta$_1$ region. This region contained 4 lines. The beta$_1$ I line was a short line below the antigen well. The protein giving this line was found in the bottom fractions when serum was subjected to ultracentrifugation. These fractions also contained antibody activity, the beta$_1$ I line may therefore represent gamma-M globulin. The beta$_1$ II line was a thin line near the antigen well, only its cathodic part was easily discernible.

Below the beta$_1$ II-line there were two lines, the beta$_1$ IV line being slightly more cathodic than the beta$_1$ III line. These lines were often faint. The beta$_1$ IV line was found in the same fractions as albumin when whole serum was subjected to ultracentrifugation or gel filtration.

Beta$_2$ region. In this region there were three lines. The beta$_2$ I and the beta$_2$ III line joined the gamma line and will be discussed later. The beta$_2$ II line was a line of heavy precipitation and represented transferrin as shown by autoradiography. Transferrin was found in the fractions containing albumin after gel filtration or ultracentrifugation of serum.

Gamma region. This region contained one heavy, almost straight line which passed through the beta region, became more curved beneath the antigen well and ended in the alpha$_2$ region between the alpha$_2$ I and the alpha$_2$ III line. The beta$_2$ I- and the beta$_2$ III lines both joined the gamma line and together they passed as a single line until it split into three in the alpha$_2$ region.

Antibody activity

After gel filtration of hedgehog antiserum to sheep red blood cells the agglutinating activity showed two maxima corresponding to the first and the second protein peak respectively. So far it has not been possible to correlate the activity of the first peak with any specific line in the immunoelectrophoretic picture. The agglutinating activity found in the first peak after gel filtration and the activity found in the bottom fractions after ultracentrifugation of hedgehog serum disappeared after treatment with mercaptoethanol. Therefore it probably represents the equivalent of the activity of gamma M of human serum.

The serologically active fractions of the second protein peak gave the beta$_2$ I, the beta$_2$ III- and the gamma line in immunoelectrophoresis. Since the proteins giving these lines appear together after gel filtration or ultracentrifugation of serum, serological activity could not be assigned specifically to any one of these lines on the basis of the present experiments. Experiments currently in progress will probably delineate the antibody function of these proteins. The protein giving the
line is probably equivalent to the gamma G of human serum and the

the beta$_2$ m-proteins may be equivalent to the gamma A and gamma-D of human serum

Discussion

The results obtained show that the protein picture of hedgehog serum does not differ greatly from that of human serum In the paper electrophoretic pattern, the most striking difference was the well defined beta$_2$-band representing transferrin in hedgehog serum The presence of haptoglobin in the beta$_1$-region and the absence of a beta lipoprotein band was also characteristic of hedgehog serum

The relative values found for the protein fractions of hedgehog serum in this study and the value for total serum proteins differ from the values found by Biorck *et al* (1956), especially in the albumin and gamma-globulin fractions The discrepancy may be based on differences in the technique employed The values agree rather well with those found by Eliassen (personal communication) using a technique similar to ours in a study on the serum of 29 non hibernating hedgehogs

Dabrowski and Skoczen (1962) studied the serum proteins of another insectivore, the mole (*Talpa europaea L*) On paper electrophoresis they found six bands, albumin, alpha$_1$, alpha$_2$, unnamed, beta and gamma Presumably it would be correct to compare the fourth and fifth bands of mole serum with the beta$_1$- and beta$_2$-bands of hedgehog serum In the hedgehog, transferrin is a beta$_2$-globulin as shown by autoradiography In the mole, the beta$_2$-band may consist mainly of transferrin since this band represents the third largest fraction of the serum protein, being exceeded by the albumin and gamma fractions The transferrin of mouse serum is a beta$_2$ globulin as shown by autoradiography (Clausen *et al* 1960) In most other mammals studied, transferrin is a beta$_1$-globulin (Haurowitz 1963) From their paper electrophoretic studies of serum proteins from a large number of mammalian species, Johnson and Wicks (1959) concluded that a comparison of electrophoretic patterns of serum proteins is of value only within a genus

The lipoproteins also differed from their human counterparts in the immuno electrophoretic patterns, human serum giving lines of "slow", 'rapid" and alpha$_1$-lipoproteins (Uriel 1958) In fresh hedgehog serum only "rapid" and slow" were found When hedgehog serum was stored for some time (months) and electrophoresis performed at room temperature, part of the "rapid" lipoprotein transformed to an alpha$_2$-lipoprotein different from the "slow" lipoprotein This transformation was reversible when the serum was cooled and electrophoresis performed at 4 C Uriel reported an observation which is pertinent to our results He found that the "rapid" (prealbumin) and the alpha$_1$-lipoproteins of human serum were immunochemically identical The amount of 'rapid" lipoprotein increased with storage although the total amounts of ' rapid ' and alpha$_1$-lipoprotien remained unchanged Accordingly, the transformation of lipoproteins in the two species sera occurs in opposite directions Whether this change has any biological significance is so far not known Since it seems to be a result of storage of the serum it will be of interest to know what influence, if any, it has on serum composition during hibernation

Another difference between the immunoelectrophoretic patterns of human and hedgehog serum was that the most conspicuous macroglobulin of hedgehog serum was an alpha$_1$- not an alpha$_2$ globulin Neither the alpha$_1$ I- nor the alpha-2 II macroglobulin found in our study could be identified with certainty as the alpha$_2$-macroglobulin found in hedgehog serum by Picard *et al* (1966)

In the immunoelectrophoretic pattern of mouse serum, Clausen (1960) found two beta lines which joined the gamma line, a situation resembling that of hedgehog serum He found that the proteins giving these lines behaved as gamma globulin in salting out experiments In our experiments with hedgehog serum we were not able to separate the beta$_2$-globulins in question from each other or from the gamma globulin The physico chemical and immuno chemical similarity to gamma-globulin gives reason to assume that these two beta$_2$-globulins may be immunoglobulins

The results of our preliminary immunization experiments with hedgehogs indicate a primary response of 19S antibodies followed by the development of 7S antibodies, a response similar to that of most other mammals Accordingly the hedgehog is well suited for studies on the effect of temperature variation on the immune response Experiments are currently in progress intended to reveal various aspects of the influence of hibernation on the primary and secondary immune response

The work was supported in part by a grant from L Meltzers hoyskolefond Bergen Norway

References

BIORCK G B JOHANSSON and S VEIGE Some laboratory data on hedgehogs hibernating and nonhibernating *Acta physiol scand* 1956 *37* 281—294

CLAUSEN J *O ersigt over immunelektroforesens teoretiske grundlag og praktiske an endelse med særligt henblik på serumproteiner* Dansk Videnskabs Forlag 1960

CLAUSEN J R RASK NIELSEN, H E CHRISTENSEN and T MUNKNER Two transplantable mouse hepatom[illegible]
Cancer Re[illegible]

DABROWSK[illegible]
mole (7[illegible]

FAHEY J and C McLAUGHLIN Preparation of antisera specific for 6 6 S globul ns β_{2A} globulins , macroglobulins and for type I and II common globulin determinants *J Immunol* 1963 *91* 484—497

FLODIN P and [illegible]
B ophys[illegible]

FUDENBE[illegible]
hemoly[illegible]

HALROWITZ [illegible]

JOHNSON[illegible]
t ons[illegible]

KABAT[illegible]

PICARD J J G VANDEBROEK J F HEREMANS and G DEFOSSE Stud es on α_2 macroglobu n III Isolation and properties of *Erinaceus α* macrogl bul n *B ochim B ophys Acta* Amst 196[illegible] *117* 111—114

URIEL J Interprétation quantitative des resultats apres electroph rese en gélose II Les proteines et les l poprotéines du sérum huma n n rmal *Cl n chim Acta* 1[illegible] 8 *3* 384 39[illegible]

SMITH I (Ed) *Chromatog aphic and ele t opho etic te hn ques* Vol II *Zone electrophoresis* W ll am Heinemann Medical books Ltd London 1960

SUOMALAINEN P and E KARPPANEN Einfluss des Winters hlafes auf das Albumin Gl bul nver haltn s des Igelserums *Suom Kem* 19[illegible] 2[illegible] 4

Acta physiol. scand 1967. 69. 270—275

From the Institute for Medical Biochemistry, University of Oslo, Norway

Glycogen Phosphorylase: A Specific Secondary Target System for Adrenergic Stimulation in the Heart

By

Ivar Oye

Received 19 October 1966

Abstract

Oye, I. *Glycogen phosphorylase: A specific secondary target system for adrenergic stimulation in the heart.* Acta physiol. scand. 1967. 69. 270—275.

Glycogen phosphorylase (α-1,4-glucan orthophosphate glucosyltransferase, EC 2.4.1.1) was studied in the isolated rat heart in the asystolic and the working state. Increasing work loads did not affect the amount of phosphorylase *a*. Anoxia was found to increase the levels of phosphorylase *a*, but this effect could be completely prevented by pretreatment with adrenergic beta receptor blocking agents. These agents also lowered the levels of phosphorylase *a* in the heart *in vivo* to levels close to those found in the isolated perfused heart. It is concluded that the phosphorylase transformation system in the rat heart under physiological conditions serves as a specific secondary target for adrenergic stimulation.

Muscle glycogen phosphorylase (α-1,4-glucan orthophosphate glucosyltransferase, EC 2.4.1.1.) exists in two separate forms (Green and Cori 1943). One of these has high catalytic activity in the absence of added cofactors and has been called "active phosphorylase" or phosphorylase *a*. The other form (phosphorylase *b*) is virtually without catalytic activity when assayed in the absence of cofactors, but is active in the presence of adenosine monophosphate (5'-AMP). Studies on crude muscle extracts as well as purified phosphorylase preparations have shown that the two forms are enzymatically interconvertible (Fischer and Krebs 1955, Graves *et al.* 1960).

Previous work on the physiological role of the transformation of the enzyme from one form to the other has recently been reviewed by Haugaard and Hess (1965). The enzyme is mainly present as phosphorylase *b* in resting muscle. Increased levels of phosphorylase *a* have been found to be associated with work, hypoxia and adrenergic stimulation. The formation of phosphorylase *a* thus appears to be part of a local mechanism for increasing glycogen breakdown under various physiological conditions, notably in situations of increased demand for energy.

TABLE I Effects of work, hypoxia and adrenaline on the levels of phosphorylase *a* in isolated perfused rat hearts

Experimental group	Conditions during perfusion	phosphorylase *a* per cent of total[1]		
1	Retrogradely perfused hearts (beating without doing any pump work)	5 7	± 1 4	(4)
2	Anterogradely perfused hearts (left ventricle contracting against 75 cm perfusate)	6 2	± 0 6	(8)
3	Asystolic hearts (arrested by increasing the concentration of K^+ above 30 mEq/l)	7 3	± 1 4	(8)
4	Exposed to 5 min of hypoxia			
	A Hearts perfused without substrates	18 5	± 1 0	(4)
	B Hearts perfused with 10 mM glucose	16 3	± 0 9	(7)
5	Exposed to 5 min of hypoxia in the presence of propranolol.			
	A Hearts perfused without substrates	7 5	± 1 1	(4)
	B Hearts perfused with 10 mM glucose	6 6	± 0 4	(4)
6	Adrenaline added			
	A Hearts perfused without substrates	16 9	± 1 3	(4)
	B Hearts perfused with 10 mM glucose	14 9	± 1 4	(4)

[1] Mean ± S E M Number of experiments in parantheses

In the experiments presented below phosphorylase has been studied in an isolated working rat heart preparation and in hearts of anesthesized rats The results suggest that the phosphorylase transformation system in the rat heart serves as a specific secondary target for adrenergic action rather than being part of a local intracellular homeostatic mechanism

Methods

Adult female breeder rats of a local strain were used throughout The hearts were removed under ether ane[illegible]

Results

Effect of work on phosphorylase activity The levels of phosphorylase *a* in isolated perfused hearts as measured by the method described, varied from about 5 per cent to 15 per cent of total phosphorylase activity In order to test a possible relationship between work and the levels of phosphorylase *a*, a series of hearts were perfused

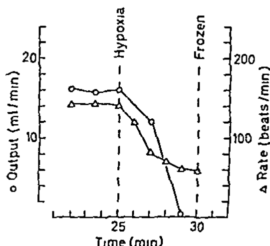


Fig 1 Effect of hypoxia on cardiac rate (—△—) and output (—○—) in an isolated perfused rat heart. At the first vertical broken line the gas supply was switched from 95 % O_2 and 5 % CO_2 to 95 % N_2 and 5 % CO_2. At the second broken line the heart was frozen for phosphorylase assay

alternately retrogradely (no pump work done) and anterogradely (working hearts). No difference in the amount of phosphorylase *a* was found (Table I, group 1 and 2). These values did not differ from those found in a group of completely asystolic hearts (Table I, group 3). Experimental asystolia in this group of hearts was achieved by substituting 10 ml of the perfusate for a similar buffer in which sodium had been replaced by potassium as previously described (Hauge and Øye 1966). In a few working hearts (not included in the table) the work load was increased to a maximum by clamping the aortic outlet tube and increasing the left ventricular filling pressure until the recorded systolic pressures reached maximal values usually about 300 mm Hg. The relative amounts of phosphorylase *a* in these strained hearts did not differ from the values found in resting cardiac muscle.

Effect of hypoxia on the levels of phosphorylase a The effect of hypoxia on the performance of the isolated working rat heart is shown in Fig 1. When the gas supply was changed from O_2, CO_2 to N_2, CO_2 cardiac output rapidly declined. Tachycardia or arrhythmia as a consequence of hypoxia was not observed in these denervated heart preparations. On the contrary the response to hypoxia was bradycardia with concomitant increase in coronary flow. The force of each single contraction measured as the maximal aortic systolic pressure remained constant during the first 5—10 min of hypoxia. The levels of phosphorylase *a* were found to be elevated in hearts frozen 5 min after the gas supply had been switched to nitrogen (Table I, group 4). As seen from the table (group 5) this effect of hypoxia on phosphorylase was completely abolished in the presence of the adrenergic beta receptor blocking agent propranolol added to a final concentration of 2.5 μg/ml. DCI had effects similar to propranolol, whereas the alpha receptor blocking agent phentolamine was without effect.

Effect of adrenaline Adrenaline added to the recirculating perfusate to a final concentration of 0.25 μg/ml increased the relative amount of phosphorylase *a* in working hearts. The hearts presented in table I, group 6 were frozen 5 min after adrenaline administration. Adrenaline also increased the amount of phosphorylase *a* in asystolic

TABLE II The effect of propranolol infusion on the levels of phosphorylase *a* in hearts of anesthesized animals The femoral vein was exposed under ether anesthesia and 1 ml of saline or a solution containing 1 mg of propranolol per ml was slowly infused during the following five minutes, whereupon the thorax was opened and the hearts excised and frozen for phosphorylase assay

Infusion *in vivo*	phosphorylase *a* per cent of total		
saline	37 0	± 1 4	(8)
propranolol	11 4	± 1.2	(8)

[1] Mean ± S E M Number of experiments in parantheses

hearts In both cases the effect of adrenaline was completely blocked by propranolol (2 5 μg/ml) added 5 min prior to adrenaline DCI had an effect similar to propranolol, whereas phentolamine did not block phosphorylase activation by adrenaline It was observed that small doses of adrenaline could affect the contractile activity of the heart without causing an evident change in phosphorylase *a* levels

In some preliminary experiments hypoxia was observed to accentuate the effect of adrenaline on phosphorylase in isolated hearts Levels of phosphorylase *a* of about 70 per cent of total phosphorylase activity was found in hearts to which adrenaline was administered during the early phase of hypoxia A ten-fold increase in the dose of adrenaline under aerobic conditions was insufficient to reproduce the high levels of phosphorylase *a* found when adrenaline was given during hypoxia

Administration of propranolol in vivo High levels of phosphorylase *a* were always found in extracts from hearts frozen immediately after removal from the animal In 8 rats killed by stunning and decapitation the mean level was 35 per cent, whereas levels above 50 per cent were found in hearts rapidly excised under ether anesthesia During prolonged anesthesia the levels were observed to decline In order to test the importance of adrenergic stimulation for these high levels of phosphorylase *a in vivo*, hearts were removed from animals under ether anesthesia 5 min after the beginning of a continuous infusion of either saline or propranolol, 1 mg per ml (Table II) As seen from the table, propranolol infusion reduced the levels of phosphorylase *a* almost to the levels found in the isolated perfused heart

Discussion

Cori (1956) demonstrated a correlation between work and the amount of phosphorylase *a* in skeletal muscle This has been confirmed by Rulon *et al* (1961) and by Danforth *et al* (1962 who concluded that the conversion of phosphorylase *b* to phosphorylase *a* was coupled to the contractile process itself The muscle preparations used by these workers were frog sartorius muscles maintained under more or less anaerobic conditions In these preparations adrenaline-mediated conversion of the *b* to the *a* form was found to be a much slower process than that observed when the muscle was made to contract under tetanic stimulation

The phosphorylase transformation system in the heart resembles that of skeletal muscle (Hammermeister *et al* 1965) It would, therefore, be expected that the amount of phosphorylase *a* would be relatively high in the continuously working cardiac muscle High levels of phosphorylase *a* in the rat heart were found by Cori (1956) However, subsequent studies on isolated perfused rat heart preparations have generally revealed low levels of phosphorylase in the *a* form Low levels have also been found in the heart of the dog and the rabbit (Krause and Wollenberger 1965) and in the working heart of the turtle (Reeves 1964)

With regard to the isolated rat heart, the standard perfusion technique previously used allowed the heart to contract but not to perform a physiological type of pump work In the present experiments an effort was made to reveal a possible phosphorylase transformation from *b* to *a* when increased work loads are imposed on the isolated heart No significant difference was found upon comparing asystolic hearts, hearts beating without doing any pump work, hearts pumping against a mean pressure of 75 cm H_2O and finally a group of hearts maximally strained by clamping the aortic outlet tube It is concluded that work *per se* does not affect the phosphorylase transformation reactions in the rat heart under aerobic conditions

Increased levels of phosphorylase *a* under hypoxic conditions have been reported by previous workers (Klarwein *et al* 1961, Parmeggiani and Morgan 1962 Cornblath *et al* 1963) This is confirmed in the present experiments Conceivably, activation of phosphorylase by hypoxia might serve as a homeostatic mechanism for increasing the rate of glycogen mobilization during conditions of insufficient oxygen supply Krause and Wollenberger (1965) found that reserpinization or treatment with pronethalol reduced the levels of phosphorylase *a* in ischemic canine hearts from 60 to 20 per cent of total phosphorylase activity They concluded that phosphorylase activation during hypoxia was partly due to adrenergic action In rat hearts excised after opening the thorax under eter anesthesia, propranolol infusion caused a similar reduction in the level of phosphorylase *a* (from 37 to 11 per cent) The 'normal levels of rat heart phosphorylase *a in vivo* is unknown and it is, therefore, not possible to judge whether or not the effect of hypoxia is completely or only partly inhibited by adrenergic blocking agents However, the experiments presented above show that hypoxia is without effect on phosphorylase in isolated hearts treated with adrenergic beta receptor blocking agents It is concluded, therefore, that the effect of hypoxia on phosphorylase transformation in some way is mediated through adrenergic beta receptors Release of noradrenaline from isolated rat hearts during hypoxic conditions has been reported by Wollenberger and Shahab (1965)

The effect of adrenaline and noradrenaline on the levels of phosphorylase *a* is well documented (Sutherland 1951 Cori and Illingworth 1956, Ellis *et al* 1957, Hess and Haugaard 1958 The fact that this effect is manifest in asystolic as well as in working hearts indicates that phosphorylase transformation after adrenaline is not secondary to the changes in physical activity *Vice versa*, it was shown in a previous paper that the inotropic and chronotropic responses were not secondary to phosphorylase transformation (Oye 1965) It is, therefore, concluded that phos

phorylase transformation from b to a is unrelated to the changes in physical activity and thus can be regarded as an independent expression of adrenergic stimulation. It is further suggested that the phosphorylase transformation system in the rat heart under physiological conditions serves as a specific secondary (intracellular) target system for adrenergic action

The skilful assistance of Mrs Marit Haug was made possible by a grant from Anders Jahres Medisinske Fond The perfusion equipment was provided by Nasjonalforeningen, Det Norske Rad for Hjerte og Karsykdommer

References

Cori C F, Regulation of enzyme activity in muscle during work *The Proteins Units of Biological Structure and Function* (O H Gaebler, ed) Academic Press, New York and London 1956 573—583

Cori G T and B Illingworth, The effect of epinephrine and other glycogenolytic agents on the phosphorylase a content of muscle *Biochem biophys Acta* (Amst) 1956 *21* 105—110

Cornblath M, J Randle A Parmeggiani and H E Morgan, Regulation of glycogenolysis in muscle effects of glucagon and anoxia on lactate production, glycogen content and phosphorylase activity in the perfused rat heart *J biol Chem* 1963, *238* 1592—1597

Danforth, W H, E Helmreich and C F Cori, The effect of contraction and of epinephrine on the phosphorylase activity of frog sartorius muscle *Proc nat Acad Sci* (*Wash*) 1962 *48* 1191—1199

Ellis S J McC [illegible] phosphate [illegible]

Fisher E H [illegible] *J biol Che*[illegible]

Graves D J [illegible] Krebs, Specificity studies on muscle phosphorylase phosphatase *J biol Chem* 1960 *235* 805—809

Green, A A and G T Cori Crystalline muscle phosphorylase I Preparation, properties and molecular weight *J biol Chem* 1943 *151* 21—29

Hammermeister, K E, A A Yunis and E G Krebs Studies on phosphorylase activation in the heart *J biol Chem* 1965 986—991

Haugaard N and M E Hess Actions of autonomic drugs on phosphorylase activity and function *Pharmacol Rev* 1965 *17* 27—69

Hauge A and I Øye, The effect of adrenaline on the oxygen consumption of perfused asystolic rat hearts *Acta physiol scand* 1966 *68* 295—303

Hess M R and N Haugaard The effect of epinephrine and aminophylline on the phosphorylase activity of perfused contracting heart muscle *J pharmacol* 1958 *122* 169—175

Klarwein M, Kako, A Chrysohou and R Bing Effect of atrial and ventricular fibrillation and ventricular tachycardia on carbohydrate metabolism of the heart *Circulat Res* 1961 *9* 819—825

Krause E G and A Wollenberger Über die aktivierung der phosphorylase und die glykolyserate im akut anoxischen hunde hertzen *Biochem Z* 1965 *342* 171—189

Morgan H E, J R Neely R E Wood C Liebecq H Liebermeister and C R Park, Factors affecting glucose transport in heart muscle and erythrocytes *Fed Proc* 1965 *24* 1040—1045

Øye I The action of adrenaline in cardiac muscle I Dissociation between phosphorylase activation and inotropic response *Acta physiol scand* 1965 *65* 251—258

Parmeggiani A and H E Morgan Effect of adenine nucleotides and inorganic phosphate on muscle phosphorylase activity *Biochem biophys Res Commun* 1962 *9* 252—256

Reeves R B, Phosphorylase activity and glycogenolysis in the working turtle heart *Amer J Physiol* 1964 *206* 898—903

Rulon R R, D D Scottelius and B A Scottelius Effect of stimulation on phosphorylase levels of excised anterior tibial muscles of the mouse *Amer J Physiol* 1961 *200* 1236—1238

Sutherland E W, The effect of the hyperglycemic factor and epinephrine on enzyme systems of liver and muscle *Ann NY Acad Sci* 1951 *54* 693 —[illegible]

Wollenberger A and L Shahab Anoxia induced release of noradrenaline from the isolated perfused rat heart *Nature* Lond 1965 *207* 89—91

Acta physiol scand 1967 69 276—283

From the Department of Pharmacology Karolinska institutet Stockholm Sweden

Ionic Binding of Histamine in Mast Cell Granules

By

CARL HUGO ÅBORG, JOSEF NOVOTNY and BÖRJE UVNÄS

Received 20 October 1966

Abstract

ÅBORG C-H, J NOVOTNY and B UVNÄS Ionic binding of histamine in mast cell granules Acta physiol scand 1967 69 276—283

the granules remains to be decided

Several *in vitro* studies have demonstrated the ability of purified heparin to form a complex with histamine The stability of the complex in distilled water even at a low pH (2—3) its instability in salt solutions as well as the inability of desulphated heparin to bind histamine indicate an electrostatic binding of the amine to the strongly acid sulphur containing groups of the heparin Amann and Werle 1956 Werle and Amann 1956 Keller 1958 Sanyal and West 1959 Kuttner 1961 Kobayashi 1962) Accordingly the high heparin content of the mast cell granules has aroused speculations about the mast cell histamine being stored as a heparin histamine complex The partial or total breakdown of the granular storage mechanism in salt solutions resembles the effect of cations on the heparin histamine *in vitro* complex Uvnäs 1964, Uvnäs and Thon 1965 Thon and Uvnäs 1966 Benditt and Lagunoff 1964 McIntire 1956 Keller 1958

It has been assumed that also intragranularly the histamine is linked to the strongly acid group of the heparin However in the mast cell granules the heparin is linked to protein forming at least in the rat mast cells a water insoluble complex The nature of this heparin protein complex has not been intensively studied but the linkage of the heparin to a protein might very well modify its histamine binding properties

Following indications obtained from previous work in our laboratory mentioned above the present studies were performed in order to elucidate the nature of the electrostatic binding of the histamine to the granular matter. The data obtained agree with an ionic linkage of the histamine to weak acid groups — COO⁻ groups — and not to SO_3 groups as deduced from the above mentioned *in vitro* observations. In fact the histamine may not be linked to heparin at all but to the protein component of the granular structure.

Experimental procedures

Isolation of mast cells

Male Sprague — Dawley rats weighing 400—500 g were anesthetized with ether and bled by cutting the carotids. 9 ml isotonic NaCl solution adjusted to pH 6 with 10 per cent Sorensen phosphate buffer was injected into the rat abdomen. After careful massage for one and a half minute the abdomen was opened along the midline. The intestines were moved aside and the abdominal fluid was sucked off with a drop pipette.

Mast cells were isolated from the wash fluid by differential centrifugation in Ficoll as described previously (Uvnäs and Thon 1961) with the minor modification that only two layers of polysaccharide (30 and 40 per cent by weight) were used. In order to minimize the contamination with inorganic salts and other ionized material after the regular washing in salt albumin solution the mast cells were washed once in isotonic sucrose solution at 0° C. Care was taken not to prolong unnecessarily the exposure of the mast cells to sucrose since the cells tended to shrink and to lose granules and histamine even when suspended in isotonic sucrose.

Owing to the tendency of both intact mast cells and mast cell fragments to adhere to glass especially when suspended in an ionfree medium (distilled water or sucrose solution) as many procedures as possible were performed in plastic tubes.

Isolation of mast cell granules

Mast cells were disrupted by suspension in distilled water for 15 min at 37° C, usually about [illegible] 10 cells in [illegible] ml of water. By differential centrifugation of the lysed cell material [illegible] 10 min at 90 [illegible] g and 20 min at 2700 × g two sediments were obtained, the heavier containing clusters of granules adhering to cell fragments, the lighter consisting of "free" granules. With a few exceptions all experiments were performed on the "free" granules fraction.

Preparation of resins

Amberlite IRC-50 (XE-64) was converted into sodium form by shaking in 1M NaOH. After filtration on a Buchner funnel washing with deionized water and air drying the resin was converted into the hydrogen form by shaking in 2N HCl. After filtration on the resin was washed in a Buchner funnel with deionized water until the pH of the effluent remained constant (pH 6 [illegible] which was the pH of our deionized water). Following the washing the resin was air dried and kept in vacuo.

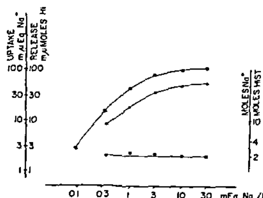
Release and uptake studies on granules

Granules from the 2700 [illegible] g sediment [illegible] above [illegible] were suspended in deionized tap water or glass distilled water and aliquots were transferred [illegible] plastic pipettes [illegible] polypropylene tubes (41 [illegible] 12 mm) with plastic stoppers. [illegible] centrifugation [illegible] 00 [illegible] g [illegible] and careful decantation 2 ml of incubation fluid [illegible] g [illegible] Na was added, the sample carefully stirred and incubated for 10 minutes. After incubation the granules were spun down at [illegible] 00 [illegible] g. The supernatant carefully decanted and radioactivity [illegible] sediment [illegible] calculations due to incomplete decantation of non granular incubation [illegible] contaminating the granular sediments were minimized by a series [illegible] different steps [illegible] experimental procedures. Since the weights of the granules were [illegible] in [illegible] granular [illegible] was also corrected for.

Release and uptake studies on resin

0.1 mg of resinous material was [illegible] incubation fluid and incubated with gentle shaking for 1[illegible] After incubation the resinous material was sedimented by centrifugation at [illegible] supernatant carefully removed by suction with a syringe. Weight e[illegible]

Fig 1 Sodium uptake (m eq) and histamine release (m moles) in mast cell granules suspended in sodium phosphate buffer pH 7 with admixture of ²²NaCl Incubation for 10 min at 0 C Ratio Na uptake/Hi release



Determination of sod um

The content of sodium was calculated from the radioactivity of admixed ²²NaCl The measureme were performed with a Philips P W 41119 type 03 scintillation detector with 41 18 01 well t crystal All samples were counted 5 times for 10 min The sodium values were expressed in m

Determination of histamine

Histamine was quantitatively determined in supernatants and sediments either by biological as on the atropinized guinea pig ileum or fluorimetrically using a slight modification of the techni described by SHORE *et al* (1959) The histamine values were expressed in m Mol

To deplete the granules of their histamine they were boiled for 5 min in isotonic NaCl soluti The total histamine content was determined in the supernatant after centrifugation at 2700 g 20 min as described above

Materials

Ficoll mol wt 370 000 Pharmacia Co Uppsala Sweden was dissolved in 0 9 chloride solution buffered to pH 7 with 10 per cent Sørensen phosphate buffer Af *1 per mille human serum albumin and 2 per cent glucose were added*

o-Phthal(d aldehyde puriss p a Fluka A G Buchs S G Switzerl
Amberlite IRC — 50 NE-64 The British Drug Houses Ltd , Pool

Results

Release of histamine and uptake of sodium

The suspension of histamine-containing granules in (sodium phosphate buffer pH 7) led to an immediate approaching 100 per cent when the sodium con 10 ³ M (Fig 1) The release of histamine was accon into the granules The exchange character of the t constant equivalent ratio (about 2 1) between the u of histamine occurring throughout the range of sodi

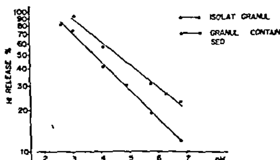


Fig 2 Influence of pH on histamine storage in mast cell granules suspended in distilled water at 0 C for 10 min pH adjusted with HCl or NaOH

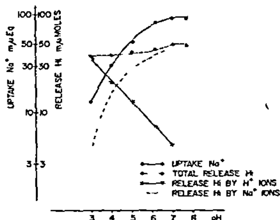


Fig 3 Influence of pH on sodium uptake and histamine release in isolated mast cell granules suspended in buffers of various pH but constant Na concentration (10 ² M)

Influence of pH on histamine retention in granules

Histamine-containing granules were suspended in distilled water and the pH was adjusted by addition of HCl or NaOH respectively The amount of NaOH required to bring pH up to 7 was negligible as regards possible cation exchange effect on the granular histamine binding sites As shown in Fig 2, the histamine content of the granules declined with falling pH practically all histamine occurring in the suspension fluid around pH 3

Influence of pH on sodium uptake and histamine release in granules

Histamine containing granules were suspended in media of various pH values but with a constant sodium concentration of 10 ² M The pH between 2 and 7 was adjusted by using mixtures of citric acid sodium chloride and NaH_2PO_4, $2H_2O$ and the pH between 7 and 8 using mixtures of boric acid, sodium chloride and sodium hydroxide Sodium uptake was calculated from the uptake by the granules of radioactive sodium from added $^{22}NaCl$

The sodium uptake was found to decrease with increasing acidity being practically abolished at pH 2—3 (Fig 3 The relase of histamine was high throughout the pH

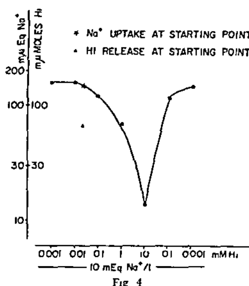


Fig 4

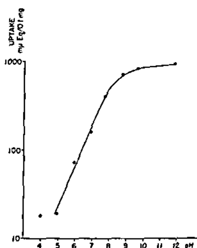


Fig 5

Fig 4 Competition between histamine and sodium for binding sites in isolated mast cell granules suspended in sodium phosphate buffer pH 7 and constant Na concentration (10^{-2} M) Histamine concentrations varied

Fig 5 Influence of pH on sodium uptake (Na^+ 10^{-2} M) in cation resin COO (Amberlite) groups Note the similarity in pH dependence between Amberlite and mast cell granules (Fig 3)

range Since the release must be due to the combined effect of hydrogen and sodium ions an attempt was made to calculate the effect ascribable to the sodium ions alone by substracting the values in the pH curve obtained from Fig 2 from the values of the total histamine release in Fig 3 As seen the calculated sodium dependent histamine release curve runs almost parallel to the sodium uptake curve, indicating the exchange character of the two processes The ratio sodium uptake to histamine release is about 2 eq Na^+/1 mole of Hi, as was the case in the experiment shown in Fig 1

Relative affinities of sodium and histamine for ionic sites in the granules

Histamine containing granules from about $1 \cdot 2 \times 10^6$ cells were suspended in a sodium phosphate buffer pH 7 containing 10^{-2} M sodium with an admixture of ^{22}NaCl Histamine was released, giving a histamine concentration in the suspension medium of $3 \cdot 4 \times 10^{-3}$ mM (black triangle Fig 4) Sodium went into the granules corresponding to a total uptake of 135 mμeq Na The granules were then spun down and the suspension fluid was changed to one with even lower histamine content (10^{-3} mM) The sodium concentration of the suspension fluid (10^{-2}M) being kept unchanged, the sodium uptake increased somewhat — up to a total of 160 mμeq From now on the granules were repeatedly spun down and resuspended in histamine-containing solutions, the histamine content being raised stepwise by a factor of ten The sodium concentration was kept constant throughout — 10^{-2} M As seen from Fig 4 the sodium uptake declined with increasing histamine concentrations It was reduced from 160 to

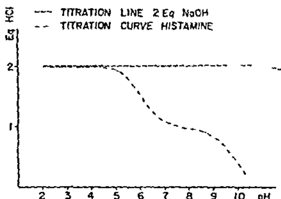


Fig 6 Titration curve for histamine

14 mμeq when the histamine concentration rose from 10^{-3} to 10 mM On the reversal of the experimental procedure i e with a stepwise reduction of the histamine concentration in the suspension medium the sodium uptake rosed and reached practically initial values indicating only insignificant losses of granular material Calculations from the exchange curve in Fig 4 suggest a ratio of about 10/1 between the affinities of histamine and sodium for ionic sites in the granules

Influence of pH on the sodium retaining capacity of carboxylic [Amberlite IRC-50 (XE 64) 0 1 mg of the resin was suspended in 2 ml of medium containing 10^{-3} M sodium buffer with an admixture of ^{22}NaCl The pH was varied as described for similar experiments on mast cell granules (p 4)

The pH curves for the sodium binding capacity of the granules and of the carboxylic resin Amberlite IRC-50 show a striking similarity (Fig 5)

Discussion

The present results confirm previous statements from our laboratory concerning the cat ionic exchange properties of granules isolated from rat mast cells The histamine release and the sodium uptake occurring on suspension of histamine holding granules in sodium-containing suspension media can be explained as due to a competition for ionic sites between histamine and sodium ions The affinity of histamine for granular sites can be calculated to be around 10 times higher than that of sodium

A noteworthy observation is the constant quantitative ratio between the uptake of Na^{+} ions and the release of histamine On the exposure of granules to sodium about two sodium equivalents replace one mole of histamine a fact which at a first sight might be taken to indicate a two point attachment of histamine to granular sites Since heparin has been suggested as the acid moiety to which histamine is bound a two-point linkage to sulphate groups might be imagined However for reasons given below, an ionic linkage betwe[illegible] within the granules

HYPOTHETICAL HEPARIN - PROTEIN - HISTAMINE BINDING

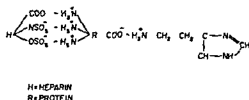


Fig 7

Isolated heparin has free sulphate groups and the *in vitro* heparin-histamine complex studied by several investigators is no doubt formed by the ionic link- age between these sulphate groups and the amino groups of histamine The titration curve for histamine (Fig 6) shows that at a pH below 4 5 histamine has two protonized amino group valencies, the amino groups being maximally ionized This fact explains the observed stability of the *in vitro* heparin-histamine complex at acid pH (3—4) At pH 7 evidently only the primary amino group — is ionized In other words, at pH 7 histamine can be ionically linked at only one point and the observed ratio 2 Eq Na^+ to 1 mole of Hi cannot be explained as due to a two point attachment of histamine at granular ionic sites

Even if purified heparin has sulphate groups available for binding of histamine, this is not necessarily or even likely to be the case for heparin in situ In the granules heparin is part of a protein heparin complex The protein component is strongly basic with a high arginine content The heparin protein linkage is probably electrostatic, since the binding is broken when the granules are suspended in 1 M salt concentration or the dissolved granules are exposed to electrophoresis As is the case with the protamine heparin complex it seems reasonable to assume that in the granules the protein-heparin complex depends on an ionic linkage between arginine imino groups and heparin sulphate groups If this is so only carboxylic acid groups should be available for the intragranular binding of histamine In fact the present observations agree well with such an assumption, the pH dependence of the histamine and sodium binding in the granules indicating linkage to weak acid groups The similarities between the binding capacity of the granules and the carboxylic acid resin Amberlite IRC-50 also favour such an assumption

Both heparin and the granular protein contain carboxylic acid groups as possible histamine binding sites In the schematic figure (Fig 7) histamine is assumed to be linked to the protein but further investigations are needed to decide to which of these groups (possibly both) the histamine is electrostatically linked

This investigation was supported by NIH Grant 5 R 01 AM 04063—06 Swedish Medical Research Council B 66—240 and funds of Karolinska Institutet

References

AMANN, R., and E. WERLE, Über Komplexe mit Histamin und anderen Di- und Polyaminen. *Klin. Wschr.* 1956, *34*, 207—209

BENDITT, E. P., and D. LAGUNOFF, The mast cell: its structure and function. *Progr. Allergy* 1964, *8*, 195—223

KELLER, R., Zur Bindung von Histamin und Serotonin in den Mastzellen. *Arzneimittel Forsch.* 1958, *8*, 390—394

KOBAYASHI, Y., Histamine binding by heparin. *Arch. Biochem.* 1962, *96*, 20—27

KUTTNER, K., *Ionenaustausch biologisch wichtiger Amine und synthetischen und natürlichen Austauschen als Modell für die pharmakologische Histaminlibera tion.* Thesis, Basel 1961, Medical Faculty, University of Bern

MCINTIRE, F. C., *The mechanisms of histamine release.* Ciba Symp. (London) 1956

SANYAL, R. K., and G. B. WEST, The histamine-heparin complex. *J. Pharm. Pharmacol.* 1959, *11*, 548—552

SHORE, P. A., A. BURKHALTER and V. H. COHN, A method for the fluorometric assay of histamine in tissue. *J. Pharmacol. exp. Ther.* 1959, *127*, 182—186

THON, I. L., and B. UVNÄS, Mode of storage of histamine in mast cells. *Acta physiol. scand.* 1966, *67*, 455—470

UVNÄS, B., Release processes in mast cells and their activation by injury. *Ann. N. Y. Acad. Sci.* 1964, *116*, 880—890

UVNÄS, B. [illegible]
Center [illegible]

UVNÄS, B. [illegible]
Exp. Cel [illegible]

WERLE, E. [illegible]
Wschr. [illegible]

Acta physiol scand 1967, 69, 284—294

From the Cardiorespiratory Research Unit, Department of Physiology, University of Turku, Finland

Response of Rat Atrium to Propranolol and Catecholamines in Various States of Thyroid Action

By

LEO HIRVONEN and T O A PAAVILAINEN

Received 26 October 1966

Abstract

HIRVONEN, L and T O A PAAVILAINEN *Response of rat atrium to propranolol and catecholamines in various states of thyroid action* Acta physiol scand 1967 69 284—294

The effect of propranolol on spontaneous beating and the response of this to adrenaline and noradrenaline was studied in hypothyroid euthyroid and thyroxine treated isolated atrium of the rat heart A significant negative chronotropic effect was noted in thyroxinized preparations in other groups this change was less significant The inotropic property remained unchanged in control conditions A propranolol dose of 1 μg/ml blocked the positive chronotropic effect of adrenaline and noradrenaline completely in euthyroid and thyroxinized atria and partially in the hypothyroid ones A competitive antagonism was observed between propranolol and catecholamines in the various preparations when contraction force and mechanical output were measured The positive inotropic effect of adrenaline and noradrenaline was highest in the hypothyroid and lowest in the thyroxinized series Only quantitative differences were observed in the responses of the atria to propranolol and catecholamines in various states of thyroid action

As a result of a quantitative comparison of the effects of sympathomimetic amines, Ahlquist 1948 presented a hypothesis concerning α and β adrenergic receptors On the basis of relative responsiveness to these substances and on the basis of specific blockade the receptor was shown to be associated with all adrenergic inhibitory effects on smooth muscle and with the adrenergic positive inotropic and chronotropic cardiac effects Ahlquist 1966 Changes in thyroid activity affect the cardiovascular system There is a mutual interaction between the catecholamines and thyroid hormones He[illegible] and Shanfeld 1965 Raab (1944) and Gross and Greenberg (1944) suggested that cardiac effects in hyperthyroidism are due to the synergistic action of thyroxine and adrenaline After giving patients with hyperthyroidism a β adrenergic receptor bl[illegible]king drug Howitt and Rowlands (1966) concluded that circulatory changes of hyp[illegible]thyr[illegible]idism are in part mediated through the sympathetic system via receptors A [illegible] conclusion was drawn by Goodkind (1966) from experiments on the guinea p[illegible] On the other hand Wilson *et al* (1966) have put

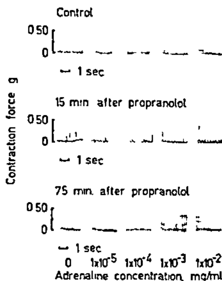


Fig 1 Mechanogram of an isolated atrium of a euthyroid rat Effect of adrenaline before and after administration of propranolol Before the last series the preparation was washed several times

forward the idea that the hemodynamic changes in man in drug-induced hypermetabolism, like those of spontaneous hyperthyroidism, are not mediated through stimulation of adrenergic receptors Cairoli (1966) noted that propranolol does not reduce the heart rate in hyperthyroid rats

The present study was planned to test the effects of adrenergic blockade on euthyroid, thyroxinized and hypothyroid heart muscle To avoid the complicating influence of the sympathetic nervous system an isolated atrial preparation was used

Methods

Ninety nine female rat
experimental animals
(British Drug) introd
daily dose of thyroxine

vertical waterfilled tube brought into direct contact with the capsule and the pressure chamber A linear relation between the loads and amplitude of the deflections was observed While the prep-

tely after the result of the previous dose had been recorded This was repeated both in th

TABLE I Maximum chronotropic and inotropic effects of adrenaline and noradrenaline in the

		Before propranolol				
		Rate of beat (per min)	Cycle length (msec)		Contraction force (mg)	
			Mean	S E	Mean	S E
Hypothyroid (n = 15)	*Control*	*78*	*770*	*216*	*120*	*26*
	Adrenaline	211	284	10	560	58
	Difference	133	486	216	440	64
	Control	98	611	82	100	17
	Noradrenaline	214	281	5	570	64
	Difference	116	330	82	470	66
Euthyroid (n = 20)	Control	175	342	23	330	48
	Adrenaline	261	230	11	490	33
	Difference	86	112	26	160	58
	Control	178	337	23	320	35
	Noradrenaline	255	235	9	490	38
	Difference	77	102	25	170	52
Thyroxinized (n = 40)	Control	269	223	6	170	21
	Adrenaline	321	187	3	280	24
	Difference	52	36	7	110	32
	Control	264	227	6	160	18
	Noradrenaline	326	184	2	260	25
	Difference	62	43	6	100	31

condition and after β receptor blockade with propranolol (Inderal I C I kindly supplied by I C I Pharmaceutical Division) The drug was given in a dose of 1 μg/ml 15 min before the administration of the test substances

Both adrenaline and noradrenaline tests were carried out twice on all the preparations The control tests were performed in alternate cases The preparation was washed with Locke s solution three or four times after each drug series and the following experiment was started after an interval of half an hour

In 6 animals of each group propranolol was injected intravenously 10 min before the heart preparation was made The adrenaline and noradrenaline tests were carried out in a similar way to those on the other preparations A preliminary report was published previously (Paavilainen and Hirvonen 1966)

Results

Original tracings of an adrenaline test on a euthyroid preparation in control conditions and 15 min and 75 min after administration of propranolol are presented in Fig 1 A positive inotropic effect of adrenaline was demonstrated both before and after propranolol After propranolol there was only a weak positive chronotropic effect

Cycle lengths, beat rates and contraction forces of the various preparations in adrenaline tests are seen as average values in Fig 2 and Table I The initial beating rate of euthyroid atria at 30 C was 175 per min The corresponding rate of thyroxi

various test series

15 min after propranolol					105 min after propranolol					Difference in cycle length before and 15 min after propranolol (msec)	
Rate of beat (per min)	Cycle length (msec)		Contraction force (mg)		Rate of beat (per min)	Cycle length (msec)		Contraction force (mg)			
	Mean	S E	Mean	S E		Mean	S E	Mean	S E	Mean	S E
64	938	256	140	34	98	609	41	100	19		
125	480	18	610	79	192	313	13	530	61		
61	*456*	*256*	*470*	*86*	*94*	*296*	*43*	*430*	*64*	*168*	*335*
86	699	62	90	17	102	588	62	140	44		
123	489	18	560	56	187	321	14	590	72		
37	210	65	470	59	85	267	64	450	84	88	103
142	422	*24*	*340*	*66*	*194*	310	*30*	240	*32*		
148	404	27	540	68	246	244	8	440	28		
6	18	36	200	94	52	66	31	200	42	80	33
144	416	29	290	46	189	317	17	290	45		
168	357	25	600	57	235	255	18	520	58		
24	*59*	38	310	73	46	*62*	25	230	73	79	*37*
206	291	8	160	18	251	239	9	160	19		
194	310	16	270	27	303	198	4	260	23		
−12	−19	18	110	32	52	41	10	100	30	68	10
199	302	*10*	*170*	*21*	*254*	*236*	*6*	*160*	*14*		
225	267	7	320	26	304	197	3	260	18		
26	35	12	150	33	50	39	7	100	23	75	12

nized preparations was 269 and that of the hypothyroid 78 per min After administration of propranolol the rate of beat decreased in all preparation groups This change, however, was not significant in hypo and euthyroid preparations

Adrenaline had a positive chronotropic effect on all preparations which was relatively strongest in the hypothyroid atria Propranolol blocked the chronotropic effect of adrenaline in euthyroid and thyroxinized preparations In hypothyroid preparations some chronotropic effect was observed after a propranolol dose of 1 μg/ml when adrenaline was administered

Fig 2 also shows the result about 105 min after the propranolol administration The preparations were washed several times As the curves show the influence of propranolol was weaker the initial rate of beat was lower than in the control series Adrenaline also had a moderate positive chronotropic effect on euthyroid and thyroxinized preparations

The initial contraction force of a single beat was strongest in euthyroid preparations and weakest in the hypothyroid ones Propranolol did not change the initial contraction force The force increased as a result of adrenaline administration in both control and propranolol tests *A comparison of control and propra*

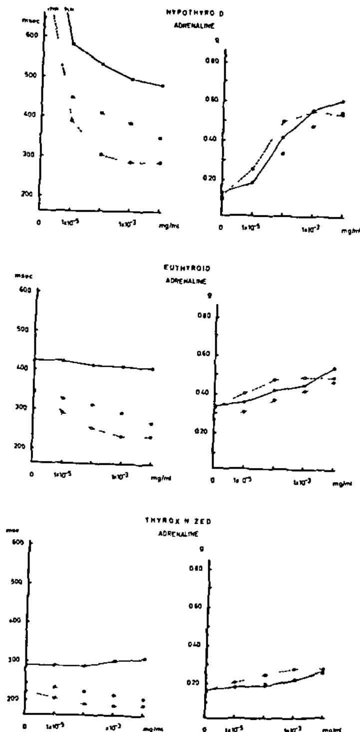


Fig 2 Cycle length (msec) and contraction force (g) of the various preparations in adrenaline tests ○– – – –○ control ●———● 15 min after propranolol (1 µg/ml) and ○ ○ 105 min after propranolol

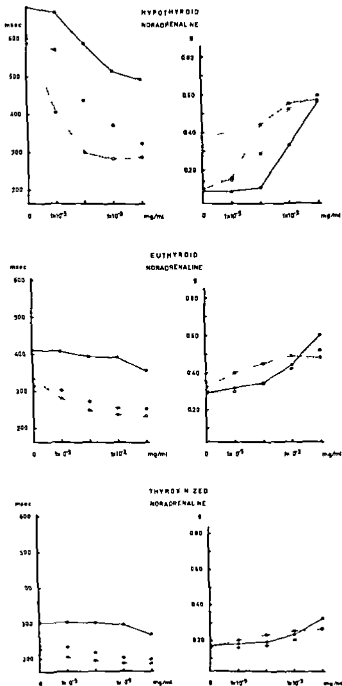
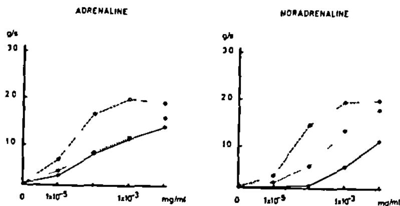
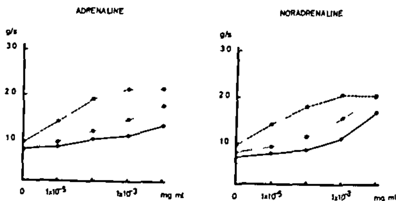


Fig 3 Cycle length (msec) and contraction force (g of the various preparations in noradrenaline tests ○— — — control ●———● 15 min after propranolol and 105 min after propranolol

HYPOTHYROID



EUTHYROID



THYROXINIZED

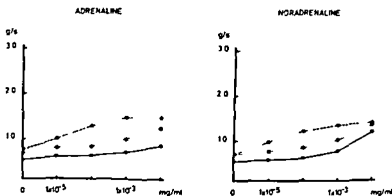
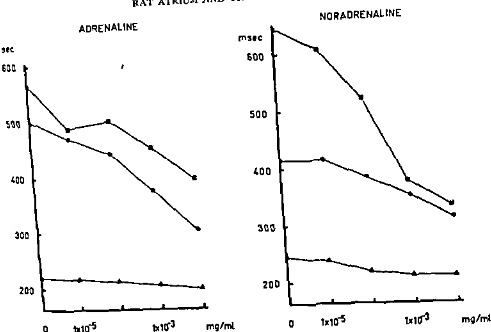


Fig 4 Mechanical output (contraction force × rate of beat g/s) of the various preparations in adrenaline and noradrenaline tests control, ●——● 15 min after propranolol 105 min after propranolol



[Fig] 3 Effect of adrenaline and noradrenaline on the cycle length of atria of the rats treated i v [wit]h propranolol (dose 1 mg kg ■ hypothyroid ● euthyroid ▲ thyroxinized)

[se]ries shows that small doses of adrenaline had a weaker effect on propranolol treated [p]reparations After large adrenaline doses the blockade was overcome The positive [in]otropic effect of adrenaline was absolutely and relatively highest in hypothyroid [a]nd lowest in hyperthyroid preparations

The results of the noradrenaline experiments are summarized in Fig 3 and Table I Noradrenaline had a positive chronotropic effect on hypothyroid preparations as did adrenaline The highest noradrenaline dose shortened the cycle length of both the euthyroid and the thyroxinized atria Propranolol partially blocked the inotropic effect of smaller noradrenaline doses The largest noradrenaline dose overcame the blockade and the contraction force was in euthyroid and hyperthyroid preparations even stronger than in the control series

Mechanical output (contraction force × rate of beat) of the atrial preparation in various experimental conditions is presented in Fig 4 The initial value was highest in euthyroid preparations Adrenaline and noradrenaline increased the output of hypothyroid preparations to the level of euthyroid ones In thyroxinized atria the maximum level remained lower The mechanical output was lower in all test series under the influence of propranolol than in the control condition Large noradrenaline doses seemed to overcome the blocking effect of propranolol more effectively than did the corresponding doses of adrenaline

Cycle lengths in [illegible]
1 mg per kg of [illegible]
had a significant [illegible] of all preparations, [illegible]

being weakest in the thyroxinized series These results indicate that the effect of an i v propranolol dose of 1 μg per g of b w was weaker than that of the same dose given *in vitro* in the bathing fluid There were no essential differences between the contraction force of these preparations, and that of those where propranolol was given *in vitro*

Discussion

Propranolol had a negative chronotropic effect on the spontaneously beating rat atrium This conforms with observations on man at rest and during exercise (Epstein *et al* 1966 Sonnenblick *et al* 1965 Howitt and Rowlands 1966) and on dog (Shanks 1966 Howe and Shanks 1966) The effect is apparently a direct action of the drug on the sinoatrial node and is similar to the effect on the atrioventricular node (Kabela and Mendez 1966) Several studies deal with the blocking influence of propranolol on the positive chronotropic effect of catecholamines especially isoprenaline in various test conditions (Glover and Hutchinson 1965 Brick and Hutchinson 1966 Wallace *et al* 1966) In the present study there was a total block of adrenalin and noradrenalin induced heart rate increase in euthyroid and thyroxinized preparations This did not occur with the hypothyroid atria when 1 μg ml of propranolol was used The mechanism of this difference cannot be identified on the basis of the present study A higher dose of propranolol may have completely blocked the effect of catecholamines The target of the propranolol effect is another problem There are plenty of sympathetic nerve endings close to the sinoatrial node in the isolated rial preparation however sympathetic nerve impulses are out of the question It would seem that stimulation of the node both by nerve impulses and directly by chemical agencies is blocked by propranolol in a similar way

The inotropic property of the atrial muscle was less sensitive to propranolol Initial levels of the contraction force before and after propranolol administration were equal in spite of the reduction of the beat rate In this respect euthyroid thyroxinized and hypothyroid preparations behaved similarly and the similarity was observed even after adrenaline and noradrenaline administration In all preparation groups the contraction force increased after propranolol treatment to a lesser degree than in control conditions when small catecholamine doses were used With larger doses (1 × 10⁻³ or 1 × 10⁻²) the block was overcome This accords well with the concept of competitive antagonism described for norepinephrine and propranolol by Benfey and Varma 1964 in the guinea pig atrium and by Nakano and Sakato (1966) in guinea pig ventricular strips

Howe and Shanks 1966 observed a negative inotropic effect in the right ventricle of the dog Brick and Hutchinson (1966) used an isolated rat heart perfused according to Langendorff Propranolol inhibited the inotropic effects of noradrenaline in their experiments In cats and dogs large doses of propranolol have been observed to elicit a direct cardiac depression unrelated to receptor blockade (Åblad *et al* 1966) In the present series this was not noted With cineangiography Wendelin *et al* (1966) noted an increase of residual and end diastolic volumes in the

dog as a result of propranolol administration while the stroke volume remained unchanged

The mechanical output of the preparation expressed as the product of beat rate and contraction force was highest in the euthyroid series, not only in control conditions (Hirvonen and Lybeck 1956) but also under the influence of propranolol. Propranolol reduced it, on an average, to half of the control value. The largest noradrenaline dose increased the output in the propranolol experiment more than the corresponding adrenaline dose.

Propranolol has qualitatively a similar effect on hypothyroid, euthyroid and thyroxinized atria. Even in the responses to catecholamines only quantitative differences were noted when propranolol was used.

This study was aided by grants from the Sigrid Juselius Foundation

References

ÅBLAD B., M BROGÅRD, B DUCE L EK, L GARBERG and B JOHANSSON, Pharmacological properties of a new beta adrenergic antagonist, H 56 28 *Acta physiol scand* 1966 *68* Suppl 277 227

AHLQUIST R P, A study of the adrenotropic receptors *Amer J Physiol* 1948 *153* 586—600

AHLQUIST R P, The adrenergic receptor *J pharm Sci* 1966 *55* 359—367

BENFEY B G and D R VARMA, Inhibition of sympathomimetic effects on the heart *Brit J Pharmacol* 1964 *23* 399—401

BRICK I and K J HUTCHINSON, The effect of a beta receptor antagonist (propranolol) on the response of the isolated perfused rat's heart to noradrenaline *J Physiol* (Lond) 1966 *183* 37P—38P

CAIROLI V J, Cardiovascular responses of the hyperthyroid rat *Fed Proc* 1966 *25* 2 Part I, 350

EPSTEIN, S E, B F ROBINSON G D BEISER and E BRAUNWALD Control of heart rate by the autonom[illegible] *Fed Proc* 1966 *25* 2 Part I 267

C[illegible] ropranolol) *J Physiol*

C[illegible] is *Clin Res* [illegible] 247

GROSS, H and H H GREENBERG The effect of thyroid extract adrenalin and a combination of these on the hearts of intact and thyroidectomized rabbits *Amer Heart J* 1944 *27* 186—202

HESS M E and J SHANFELD Cardiovascular and metabolic interrelationships between thyroxine and the sympathetic nervous system *J Pharmacol* 1965 *148* 290—297

HIRVONEN L, Electrographic studies of the isolated rabbit auricle *Ann Med exp Fenn* 1955 *33* Suppl 7

HIRVONEN L, Effect of adrenaline and noradrenaline on the isolated rat auricle in various states of thyroid action *Ann Med exp Fenn* 1956 *34* 10—115

HIRVONEN, L and H LYBECK Mechanogram observations on the isolated auricle of hypothyroid rat *Acta physiol scand* 1956 *36* 29 37

HOWE R and R G SHANKS Optical isomers of propranolol *Nature* (*Lond*) 1966 *210* 1336—1338

HOWITT G and D J ROWLANDS Beta sympathetic blockade in hyperthyroidism *Lancet* 1966 I 628—631

KABELA E and R MENDEZ Action of propranolol on the atrioventricular node and on its response to adrenaline and isoprenaline *Brit J Pharmacol* 1966 6 473—481

NAKANO J and M SAKATO Competitive antagonism between norepinephrine and propranolol *Experientia* (Basel) 1966 [illegible] 112 113

PAASONEN T O A and L HIRVONEN Response of propranolol pretreated isolated atrium of a hypothyroid and thyroxinized rat heart to adrenaline and noradrenaline *Acta physiol scand* 1966 *68* Suppl 277 155

RAAB W Epinephrine tolerance of the heart altered by thyroxine and thiouracile *J* [illegible] 1944 *82* 330—333

SHANKS, R. G., The effect of propranolol on the cardiovascular responses to isoprenaline, adrenaline and noradrenaline in the anaesthetized dog. *Brit. J. Pharmacol.* 1966. 26. 322—333.
SONNENBLICK, E. H., E. BRAUNWALD, J. F. WILLIAMS, JR. and G. GLICK, Effects of exercise on myo-

Acta physiol. scand. 1967. 69. 295—303

From the Institute of Medical Chemistry, University of Helsinki, Finland

Mechanism of Calcium and Adenosine Triphosphate Induced pH Changes in Sarcotubular Membrane Fractions from Rabbit Skeletal Muscle

By

ULLA-PIA HERMIÖ and NILS-ERIK SARIS

Received 26 October 1966

Abstract

HERMIÖ, U.-P. and N.-E. SARIS. *Mechanism of calcium and adenosine triphosphate induced pH changes in sarcotubular membrane fractions from rabbit skeletal muscle.* Acta physiol. scand. 1967. 69. 295—303.

Addition of calc[illegible]
and A[illegible]
sequer[illegible]
substa[illegible]
inorga[illegible]
was de[illegible]
ATP [illegible]
aminohydrolase (E.C. 3.5.4.6). The highest specific activity of AMP aminohydrolase was found in the sarcotubular membrane and light membrane fractions. Accumulation of calcium in conditions where binding by mitochondria was excluded, was used as a biochemical marker for sarcotubular membranes. These findings are discussed in relation to pH changes observed in muscular activity and as a mechanism of preventing excessive drops in muscular intracellular pH.

Addition of calcium to a mitochondrial suspension in appropriate conditions gave rise to a pH cycle (Saris 1959). It consisted of a pH drop due to ejection of hydrogen ions during accumulation of the added calcium by the mitochondria and of a pH rise associated with a disintegration of mitochondrial structure and function (Saris 1963). Though the mechanism of the hydrogen ion ejection is not completely clarified, formation of concentration gradients of cations and hydrogen ions across the mitochondrial inner membrane is now generally believed to be primarily involved (see review by Chance 1965).

In this study the occurrence of a similar pH cycle in the calcium accumulating system derived from the sarcoplasmic reticulum of skeletal muscle (Ebashi 1961, Hasselbach and Makinose 1961, 1963) is reported. [illegible]

responsible for this effect has been the aim of the present investigation Preliminary results have been reported elsewhere (Herniö and Saris 1966)

Methods

Adult male rabbits weighing 2—3 kg were stunned by a blow on the neck and bled to death Adductor longus muscles were excised, placed in ice cold 0 3 M sucrose cut to pieces and homogenized for 20 sec using an Ultra Turrax homogenizer, type TP 18/2 (Janke & Kunkel KG, Staufen, West Germany,

The fraction (H_M) containing sarcoplasmic reticulum fragments was obtained by differential centrifugation (50 min 30 000 x g) after discarding debris (7 min 1 000 x g) and mitochondria (15 min 10 000 G and 15 min 20 000 x g) A lighter membrane fraction (L_M) was harvested between 50 000 x g (50 min) to 105 000 x g (60 min) The pellets were suspended in 0 3 M sucrose and kept in ice until used

Protein and inorganic phosphate (P_i) estimations and measurements of pH were carried out as described previously (Saris 1963) For the estimations of ammonia the method of Humoller et al (1964) was used, and the determination of succinate tetrazolium reductase (E C 1 3 99 1) was done according to Pennington (1961) Myosin was estimated by means of the ATP phosphohydrolase activity elicited by 1 mM EDTA in a medium containing 0 5 M KCl 5 mM ATP, and 100 μM N, N-dicyclohexylcarbodiimide (DCCD) 1 mM EDTA completely inhibits microsomal ATP phosphohydrolase activity while mitochondrial activity is blocked by DCCD (Beechey et al 1966) ATP AMP phosphotransferase activity was determinated by measuring the ATP phosphohydrolase activity in a medium containing ADP

All incubations were carried out at room temperature (22—24°C) The incubation medium used in the calcium experiments was 100 mM KCl 25 mM tris HCl buffer pH 6 8, 4 mM $MgCl_2$, 4 mM trisoxalate and 5 mM ATP In the Triton experiments oxalate was omitted The medium in the AMP experiments consisted of 100 mM KCl, 25 mM tris HCl buffer pH 6 8 and 4 mM $MgCl_2$. The incubation medium in the ATP AMP phosphotransferase activity measurements was identical with that in the calcium experiments, except that ADP was substituted for ATP In calcium, Triton and AMP aminohydrolyse experiments there was 1 mg protein per ml ATP phosphohydrolase and ATP AMP phosphotransferase determinations were carried out in the presence of 0 2 mg protein per ml

ATP, ADP and 5'-AMP were obtained from Boehringer und Soehne IMP cyclic 3' 5' AMP and tris(hydroxymethyl)aminomethane (tris) from Sigma Chemical Co and 2,4 dinitrophenol (DNP) from Merck DCCD was obtained from Fluka and ethylene glycol bis (β amino-ethylether) N N'-tetra acetic acid (EGTA) from Geigy All solutions were neutralized with tris

Results

pH cycles recorded on several additions of calcium chloride corresponding to final concentrations of 300 μM are shown in Fig 1 From the amplitude of the pH cycle and by appropriate titration of a pH change with a hydrochloric acid solution the H^+/Ca^{++} ratios can be calculated Values up to 0 5 were found in different prep-

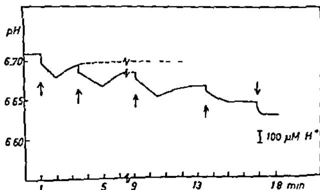


Fig 1 pH cycles induced by sequential additions of calcium to a H_M particle suspension
↑ Addition of 300 μM Ca^{++}
↓ Addition of EGTA

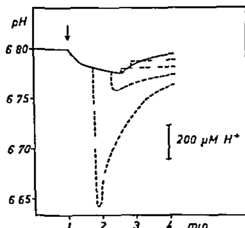


Fig 2 pH responses to additions of EGTA during the calcium induced pH cycle
↓ Addition of 500 µM Ca
— pH record of the pH cycle
pH record after addition of EGTA

arations when the small pH drop obtained on addition of calcium to the medium in the absence of H_M particles was corrected for

The pH rise begins when the added calcium has been removed from the medium by the sarcoplasmic reticulum particles The amount of free calcium ions present in the medium can conveniently be measured by the immediate pH drop obtained on addition of an excess of EGTA, which will chelate calcium with the liberation of two hydrogen ions per one calcium ion Fig 2 shows the pH records obtained when EGTA is added at various points of the calcium induced pH cycle The small upward deflection of the trace seen when EGTA is added at the pH rising phase was due to a slight difference in pH between the EGTA solution and the medium Following the EGTA induced pH drop there often was a pH rise which probably was due to the ability of the particles to accumulate calcium from extremely low concentrations (2×10^{-9} M Makinose and The 1965) thus being able to remove calcium from its complex with EGTA

This interpretation is strengthened by the finding that when calcium was introduced after EGTA, a pH rise again followed the initial drop The EGTA method may still be used for the estimation of the point where virtually all calcium has been removed from the solution even though the estimation of the concentration of calcium becomes somewhat involved By extrapolation of the rising pH trace to the point of addition of EGTA a measure of the hydrogen ion production in chelation of free calcium ions can still be obtained Another limitation of the EGTA method is encountered when most of the calcium binding capacity of the H_M particles has been used up Then EGTA seems to be able to successfully compete with the particles for calcium After an initial pH drop on addition of EGTA reflecting a binding of free calcium ions a continued slow pH drop is seen as if calcium continually were lost from the particles

In the mitochondrial system breaking the membranes with a detergent such as Triton X—100 after the calcium induced pH drop promptly evoked a pH rise (Chappell *et al* 19

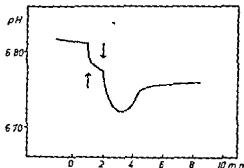


Fig. 3. Triton induced pH cycle at the point of maximal pH drop caused by calcium
↑ Addition of 300 μM Ca^{++}
↓ Addition of 0.1% Triton

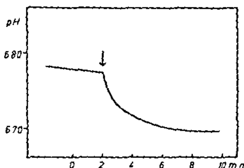


Fig. 4. pH cycle induced by Triton in the abscence of magnesium
↓ Addition of 0.1% Triton

— a further pH drop — was found (Fig. 3). Addition of Triton X—100, with or without a previous addition of calcium, also caused a complete pH cycle which had an even greater amplitude than the one obtained with calcium (Fig. 3 and 4). Without ATP no pH change was seen. If magnesium was omitted the pH drop was slower and the pH rise absent or very slow (Fig. 4). These findings exclude gradient formation as a mechanism for the observed pH cycles and prompted a search for enzymatic reactions in which adenine nucleotides and hydrogen ions are involved.

One reaction, in which hydrogen ions are produced, is the hydrolysis of ATP to ADP and inorganic phosphate. pH measurement may conveniently be used to follow

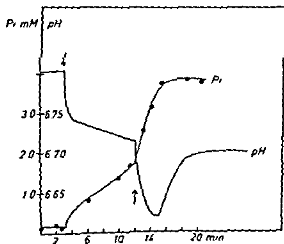


Fig. 5. ATP- and Triton induced pH changes and ATP phosphohydrolase activity in Hg particle suspension
↓ Addition of 5 mM ATP
↑ Addition of 0.1% Triton

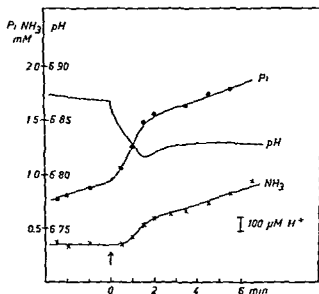


Fig 6 Calcium induced pH cycle and production of ammonia and P_i
↑ Addition of 500 µM Ca

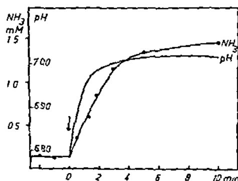


Fig 7 AMP induced changes in pH and production of ammonia in a suspension of H_{3t} particles
↑ Addition of 1 mM AMP

5 -AMP 1 IMP 2 Cyclic 3 5 -AMP

Fig 8 Schematic description of results of thin layer chromatography
1 Sample before addition of AMP
2 Sample after AMP induced pH rise

formation or hydrolysis of ATP Saris 1959 Nishimura *et al* 1961 The drop induced by addition of Triton X—100 (Fig 5) or calcium Fig 6 coincided with liberation of P_i

A pH rise was produced by addition of AMP to a H_{3t} particle suspension concomitant with production of ammonia (Fig 7) Involvement of AMP aminohydrolas

TABLE I Distribution of various activities in rabbit skeletal muscle homogenate fractions

Fraction		EDTA stimulated ATP phosphohydrolase mIU/mg prot		Succinate tetrazolium reductase Relative activity			Calcium accumulation µmoles mg prot	
Homogenate		85	—	—	71	28	—	—
1	1 000 × g	87	57	18	55	36	0 1	—
2	10 000 × g	67	38	100	100	100	2 5	0 7
3	20 000 × g	0	0	47	42	38	2 0	1 2
4	30 000 × g	0	0		8	14	2 5	1 3
4+5				16				
5	50 000 × g	0	0			6	—	0 13
5+6					0			
6	105 000 × g	0	0	14		5	0	0
Supernatnat		0	0	5	0	0	0	0

activity in the pH rise phase was further indicated by formation of inosinic acid (Fig 8) Production of ammonia after a short lag is observed also in the calcium-induced pH cycle (Fig 6)

The distribution of cellular components in the various homogenate fractions is seen in Table I EDTA-stimulated ATP-hydrolyzing activity, which is characteristic of myosin is not demonstrable in fractions sedimented at higher centrifugal forces than 10,000 *g* The H_M and L_M fractions contained only traces of mitochondrial fragments as evidenced by the low succinate tetrazolium reductase activity Maximal calcium accumulation in conditions where mitochondrial accumulation was inhibited by DCCD and DNP served as a biochemical marker for H_M material This was found also in appreciable amounts in the main mitochondrial and heavier fractions The highest specific AMP aminohydrolase activity was found in the L_M and main H_M fractions, though the highest total activity was present in the supernatant, as reported also for mouse and man (Park and Pennington 1966)

From Table I it is also evident that ATP AMP phosphotransferase activity is distributed among the various particulate fractions without being restricted to myosin or mitochondrial fragments-containing fractions The magnesium stimulated ATP phosphohydrolase activity may have been rate limiting in the assay system used, and the actual activities may have been somewhat larger In any case it is clear that the enzymes involved in the pH cycle events are present in sufficient high specific activity in the H_M particle fraction

Discussion

The calcium-induced pH cycle may be readily accounted for following events (Fig 9)

Addition of calcium activates the calcium pump of particles derived from the sarcotubular system (Hasselbach and Makinose 1961 1963, Ebashi 1961) Calcium is

AMP amino hydrolase mIU/mg prot				ATP AMP phosphotransferase mIU/mg prot		Magnesium-stimulated ATP phosphohydrolase mIU/mg prot
—	150	88	—	8	76	57
165	119	81	—	11	73	75
96	65	24	—	18	56	22
76	42	71	—	77	27	30
	235	78	49	58	30	36
145						
		54	50	23	10	88
	520					
690		305	59	7	27	30
463	142	161	22	6	7	6

removed from the medium concomitant with hydrolysis of ATP When the concentration of calcium has been reduced to a low level, stimulation of ATP phosphohydrolase activity ceases The observed drop of pH is accounted for by the burst of calcium stimulated ATP phosphohydrolase activity From the ADP thus formed, AMP is produced by the ATP AMP phosphotransferase activity present in the preparation This reaction is not associated with a change in pH it is, however, dependent upon the presence of magnesium ions AMP is converted to inosinic acid and ammonia by AMP aminohydrolase In the formation of ammonium ions hydrogen ions are consumed, which accounts for the rise in pH The actual pH changes recorded reflect changes in the rates of hydrolysis of ATP and deamination of AMP The mechanism responsible for the calcium induced pH cycle in the sarcotubular system is thus completely different from the one producing cyclic pH changes in mitochondria where gradients of hydrogen ions seem to be involved (Saris 1959 1963)

Intracellular muscular pH is probably around pH 6 8 (Bittar 1964), which is a reason why a medium of this pH was used in this study At higher pH, the hydrogen ion yield of the hydrolysis of ATP increases (Saris 1959 Nishimura *et al* 1962), but the pH cycle is still demonstrable showing a distinct pH rise or a break in the rate of production of hydrogen ions It is evident that in systems, where also AMP aminohydrolase and ATP AMP phosphotransferase are present ATP phosphohydrolase activities should not be followed by pH measurements alone The inhibition of ATP

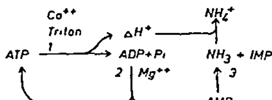


Fig 9 Schematic diagram of reactions producing the pH changes
1 ATP phosphohydrolase
2 ATP AMP phosphotransferase
3 AMP aminohydrolase

phosphohydrolase activity by calcium concentrations in excess of 100 μM reported by Martonosi and Feretos (1964) could just as well be stimulation of AMP aminohydrolase activity

In intact muscle, Jobsis (1963) and Disteche (1964) observed a pH drop followed by a rise in a muscular twitch Jobsis (1962) attributed the pH drop to glycolytic production of lactic acid and the pH rise to splitting of creatine phosphate which is associated with a rise in pH (Lipmann and Meyerhof 1930) It is possible that the reactions studied in this paper also contribute to the pH changes reported to occur in muscular activity However, definite formation of inosinic acid has been found only in extreme muscular stresss and in *rigor mortis* (Murray and Jones 1958), possibly because inosinic acid is converted to adenine nucleotides with the aid of GTP and the aminogroup of aspartic acid (Lieberman 1956, Yefimochkina and Braunstein 1959, Newton and Perry 1960, Davey 1961) As pointed out already by Lipmann and Meyerhof (1930), H^+ ion production due to glycolysis, and H^+ ion consumption due to hydrolysis of creatine phosphate are of the same order of magnitude After the exhaustion of creatine phosphate, energy for contraction must be drawn from adenine nucleotides in a H^+ ion producing process A corresponding consumption of H^+ ions may be provided in this situation by the hydrolysis of AMP The physiological role of the high AMP aminohydrolase activity in skeletal muscle may be related to the need for maintenance of a constant pH in muscle Even a temporary halting of the intramuscular pH drop due to glycolysis and other reactions in severe muscular stress may preserve muscular activity, preventing relaxation due to excessive lowering of pH (Szent Gyorgyi 1953), a little bit longer, and may thus have a distinct survival value The low pH optimum of the AMP aminohydrolase (Hermann and Josepovits 1941, Nikiforuk and Colowick 1956, Lee 1957) may be viewed as an adaptation to this function Another role could be removal of AMP, which inhibits ATP AMP phosphotransferase when ADP is the substrate (Colowick and Kalckar 1943)

Supported by grants by The Juselius Foundation and by the Finnish State Research Council for Sciences

References

Beechey R B C T Holloway J G Knight and A M Robertson The effects of dicyclohexylcarbodiimide (DCCD on energy linked reactions FEBS III Meeting Abstracts 1966 M 46

Bittar, F E, *Cell pH* Butterworths London 1964

Chance, B The energy linked reaction of calcium with mitochondria *J biol Chem* 1965 *240* 2729—2748

Chappell, J B, G D Greville and K E Bickenell Stimulation of respiration of isolated mitochondria by manganese ions *Biochem J* 1962 *84* 61 P

Colowick, S P and H M Kalckar The role of myokinase in transphosphorylations I The enzymatic phosphorylation of hexoses by adenyl pyrophosphate *J biol Chem* 1943 *148* 117—126

Davey, C I The amination of inosine monophosphate in skeletal muscle *Arch Biochem* 1961 *95* 296—304

Distèche, A, The quantitative interpretation of muscle surface pH measurements and the phosphocreatine breakdown in muscles treated with hypertonic Ringer's solution in "*Biochemistry of muscle contraction* ed by J Gergely J & A Churchill Ltd London 1964 458—462

Ebashi S, Calcium binding activity of vesicular relaxing factor *J Biochem* (Tokyo) 1961 *50* 236—244

Muskels und

durch die

1941 *164*
845—846

pH changes in skeletal muscle micro-

le II Recovery from contractile activity

the crystalline enzyme from rabbit skeletal

Lieberman I Involvement of guanosine triphosphate in the synthesis of adenylosuccinate from inosine 5 phosphate *J Amer chem Soc* 1956 *78* 251

Lipmann F and O Meyerhof, Über die Reaktionsänderung des tätigen Muskels *Biochem Z* 1930 *227* 84—109

Newton A A and S V Perry The incorporation of [15]N into adenine nucleotides and their formation from inosine monophosphate by skeletal muscle preparations *Biochem J* 1960 *74* 127—136

Nikiforuk G and S P Colowick The purification and properties of 5 adenylic acid deaminase from muscle *J biol Chem* 1956 *219* 119—129

P

Pennington
tase and

Saris N

Saris N
Physico-

Szent Györgyi A *Chemical physiology of contraction on body and heart muscle* Acad Press New York 1953 44

Yefimochkina E F and A E Braunstein The amination of inosinic acid to adenylic acid in muscle extracts *Arch Biochem* 1939 *83* 350—352

Acta physiol scand 1967, 69 304—312

From the Department of Pharmacology Karolinska Institutet Stockholm Sweden

Histamine Release from Rat Mast Cells Induced by a Mast Cell Degranulating Fraction in Bee Venom

By

Bo Fredholm and Östen Haegermark

Received 31 October 1966

Abstract

Fredholm, B and Ö Haegermark *Histamine release from rat mast cells induced by a mast cell degranulating fraction in bee venom* Acta physiol scand 1967 *69* 304—312

Bee venom is known to contain high amounts of phosphatidase A and to cause degranulation and histamine release in rat mast cells In the present study bee venom was fractionated on Sephadex G 50 whereby the phosphatidase A (Fraction F I) was separated from the histamine releasing activity (Fraction F II) Various aspects of the F II induced histamine release from rat peritoneal mast cells were studied namely the dose response curve the time course of the reaction the pH and temperature dependency and the influence of inhibitors It is concluded that this bee venom fraction acts by a mechanism similar to that of compound 48/80 This is in contrast to findings reported for melittin a histamine releasing bee venom fraction described by other authors

In the late thirties bee venom and snake venoms rich in phosphatidase A were shown to release histamine in several animal species (Feldberg and Kellaway 1937a 1937b) The phosphatidase was assumed to be responsible for this effect Later, semipurified phosphatidase A from bee venom was reported to degranulate rat mesentery mast cells Hogberg and Uvnas 1957) a finding which has been confirmed repeatedly in this laboratory The mast cell degranulating action of bee venom *phosphatidase A is blocked by anoxia and metabolic inhibitors in the same way as* that of compound 48 80 Hogberg and Uvnas 1960 Uvnas Diamant and Hogberg 1962) This led to the assumption that the bee venom preparation degranulates mast cells not by a lytic process but in a manner similar to that of compound 48/80 i e by activating an enzymatic energy dependent mechanism in the mast cell Although a few experiments have demonstrated that bee venom induces histamine release from rat peritoneal mast cells Moran Uvnas and Westerholm 1962), no conclusive investigation has been made as to its release mechanism

Even though phosphatidase A may be responsible for the venom induced histamine release in certain animal species recent findings have shown that the release *from rat periteonal* mast cells is due to other factors in the venoms A histamine releasing peptide, melittin, has been isolated and characterized by Habermann and

coworkers (for references see Habermann 1963), and its effect in the rat studied by Rothschild (1965) Fredholm (1966) has also reported that bee venom can be separated into a phosphatidase A containing fraction (F I) and a mast cell degranulating fraction (F II) However, this mast cell active fraction did not have the same properties as melittin

The aim of the present study was to ascertain whether the mechanism of the histamine release induced by bee venom Fraction F II is similar to that for compound 48 80 and whether this factor can fully account for the histamine releasing effect of the unfractionated bee venom

Methods and materials

Fract onation of bee venom

we g ed and stored in the refrigerator un [illegible]
releasing fractions were treated similarly and the product called F II Some tubes contained a mixture of both activities due to tailing of the phosphatidase A and care was taken not to include these tubes in the pools

Preparation of cell suspension

Incubation technique

age of the total amount of h stamine

All incubations were run dupl cate Spontaneous release amounted to 2 2 ± 0 4 (M ± S E M) per cent and was not deducted

Histamine a say

Histamir
(1959
berated
om tted
When the conden at n rea tion was stopped by acid fication the solution became opalescent from precipitat l the serum albumin The precipitate was removed by centrifugation at 2000 *g* for 5 min and the fluorescer ce of the clear supernatant measured

Bee enom pho phat e A in the f llowing called "bee venom prep red w th ion exchange chromatography on Amberlite IRC 50 and *compoun l 48 80* were suppl ed by Dr B Hogberg AB Leo H lsingb rg S den

Other sub tance used were bta ned from standard commercial s urces

Results

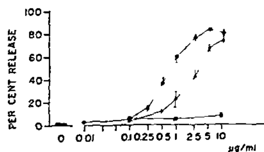
The peritoneal cell suspensions contained 3—5 per cent mast cells. It has previously been shown that these cells are the only histamine containing structures in such a suspension (Smith 1958), i. e. all the histamine released originates from the mast cells. Accordingly, identical release values were obtained when testing various concentrations of bee venom on either a pure mast cell suspension prepared as described by Uvnäs and Thon (1959), or on an unfractionated cell suspension. Having shown this we used the cell suspensions directly without isolating the mast cells.

The number of mast cells obtained from different rats varied considerably, and in some experiments it was necessary to use a suspension concentrated ten fold. It was established that such a variation of the cell concentration did not influence the percentual histamine release (Table I). Similar results have been obtained with compound 48/80 by other authors (Uvnäs and Thon 1959, Bray and VanArsdel 1961).

The fractions F I and F II of this work correspond to those described in a previous paper (Fredholm 1966), however, in the present work a volatile buffer was used for the elution in order to obtain the fractions free of salt.

TABLE I. Effects of cell concentration on histamine release induced by bee venom 1 μg/ml

Dilution of cell suspension	Histamine μg			Per cent release
	Sediment	Supernatant	Total	
1:1	11.8	10.9	22.7	48.0
	10.7	9.7	20.4	47.5
1:2	5.5	5.1	10.6	47.9
	5.4	4.9	10.3	47.6
1:10	1.18	1.26	2.34	53.8
	1.20	1.08	2.28	47.4



bars indicate standard errors. Note the absence of release by the ph... phatidase fraction.

Dose-response relationship

Cells were incubated with the releaser at 37° C for 10 min to allow the release reaction to be completed. The results are shown in Fig. 1. One μg/ml of bee venom gave approximately 60 per cent histamine release and 10 μg/ml 85 per cent. The dose-response curve for F II had the same shape as that for bee venom, but the activity per μg was less (3—5 μg/ml corresponding to 1 μg/ml of the unfractionated material). F I had no effect in the dose range used, although some release occurred with doses above 50 μg/ml.

The following experiment was performed to ascertain the reason for the decreased activity of F II. Five mg of bee venom were run through the usual Sephadex G 50 column and the total effluent volume collected in one beaker. It was freeze dried, weighed and tested for histamine releasing activity. The recovered material, 5.3 mg, weighed virtually the same as the bee venom introduced to the column. However, the histamine releasing activity of the filtered material was only about one third that of the unfiltered substance, indicating that the loss of activity reflects a real inactivation of the material during the filtration, and not a contamination with the eluant salt.

Influence of temperature

It has previously been shown that 48/80-induced histamine release from isolated rat mast cells is reversibly inhibited at low temperatures and irreversibly blocked at temperatures above 44° C (Uvnäs and Thon 1961). Mast cell degranulation induced by bee venom was also reported to be irreversibly inhibited by preheating at 45° C (Uvnäs and Antonsson 1963). In the present investigation we found that although the histamine releasing effect of moderate doses of both bee venom and F II (1.0 and 5.0 μg/ml respectively) was nearly abolished at 0° C, a ten fold increase of the dose resulted in marked release at this temperature. Preincubation of the cells for 10 min at 0° C caused a slight decrease in subsequent release at 37° C.

When the cells were preheated at 47° C for 10 min they became less sensitive to the action of both bee venom and F II. However, the release induced by F II was considerably more impaired by such treatment than was the effect of unfractionated bee venom (Table II).

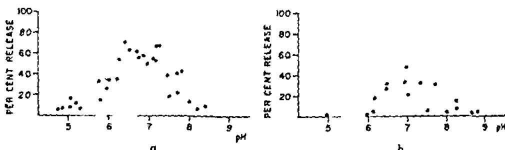


Fig 2a Influence of pH on histamine release induced by bee venom 1 μg/ml Each dot represents the mean of duplicate samples
Fig 2b Influence of pH on histamine release induced by F II, 5 μg/ml Each dot represents the mean of duplicate samples

Influence of pH

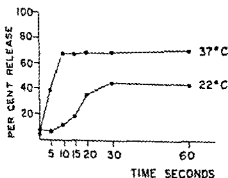
It was found that the routinely used phosphate buffer could be replaced by tris HCl buffer without altering the histamine release values Consequently the latter could be used in the pH range not covered by the phosphate buffer Both bee venom and F II were found to have a pH optimum with maximal effect between pH 6 5 and 7 5 Below and above this range a marked inhibition of the release occurred (Fig 2)

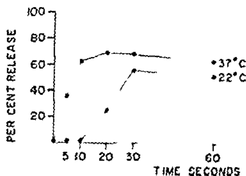
Time course of the histamine release

Histamine release from rat peritoneal mast cells is known to be rapid After exposure to compound 48/80 at room temperature the release was reported to be completed within 20 sec (Moran, Uvnas and Westerholm 1962) In order to get a better idea of the initial course of the process we developed a method to obtain release values from shorter incubation periods This method involved the termination of the reaction by rapidly diluting the sample volume ten fold with ice cold buffered salt solution Thereby the concentration of releaser was reduced to a subliminal value and the temperature brought to a point where no release occurred unless high doses of releaser were used The technique was as follows To 0 1 ml of the releaser solution 0 9 ml cell suspension was added, both solutions being separately prewarmed to the incubation temperature After the appropriate incubation time 9 ml of ice cold salt solution were rapidly pipetted into the reaction tube The tubes were quickly centrifuged at 1 2 C and handled as described under Methods Using this technique it was possible to make accurate determinations of the release during periods as short as 5 sec

The rate of release was dependent on the concentration of the liberating agent and on the incubation temperature Fig 3) With 1 0 μg ml of bee venom at 37 C the release was nearly completed after 10 sec while at 22 C there was a lag period of 5–10 sec, after which the release proceeded more slowly than at 37 C (Fig 3a) When the dose was increased to 5 μg ml the lag at 22 C was diminished to less than 5 sec, and the rate of release increased

The time course of the F II induced histamine release was similar to that of bee venom (Fig 3b)





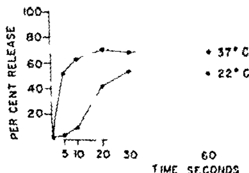


Fig 3a Time course of histamine release induced by bee venom 1 μg/ml For methodological details see text

Fig 3b Time course of histamine release induced by F II 5 μg/ml

Fig 3c Time course of histamine release induced by compound 48/80 0 3 μg/ml

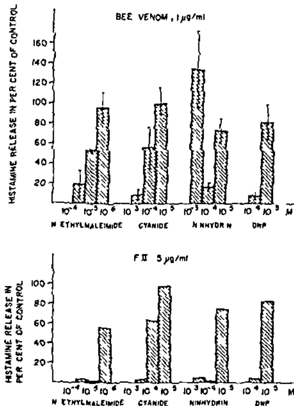


Fig 4 Influence of inhibitors on histamine release Cells were incubated with inhibitor for 30 min at 37 C before addition of releaser
a Incubation with bee venom, 1 µg/ml Each column represents the mean of 3—5 experiments Vertical bars indicate standard errors
b Incubation with F II, 5 µg/ml Each column represents the mean of 2 experiments
Spontaneous release was deducted before calculation

Discussion

Much discussion has been devoted to the relationship between mast cell degranulation and histamine release Although toluidine blue has been reported to cause histamine release without mast cell degranulation (Smith 1958), much evidence suggests that there is a close relationship between these two processes for many histamine releasing agents (Paton 1957) In agreement with this, results of recent findings on the release from isolated mast cell granules have shown that their histamine is liberated when they are exposed to cations in the surrounding fluid (Uvnas 1965) In the electron microscope it has been observed that in connection with histamine release not only degranulation occurs, but also morphological changes of the intracellular granules (Bloom and Haegermark 1965, Singleton and Clark 1965) When the present data on per cent histamine released are compared with those from earlier observations on per cent degranulated rat mesentery mast cells (Uvnas and Antonsson 1963, Fredholm 1966) a close correlation is found

The histamine releasing effect of fraction F II on the rat mast cells showed fundamental similarities to the action of compound 48/80 Many workers have reported that the effect of compound 48 80 on rat mast cells is blocked either by metabolic inhibitors or by preheating at 47 C, that it does not initiate histamine release at

0 C, and that the pH optimum for the reaction is around 7 (for references see Uvnäs 1961) All these criteria hold true also for F II

Further evidence that the same mechanisms are activated in the mast cells by F II and compound 48/80 is obtained from studies of the rate of histamine release It was observed (Moran, Uvnäs and Westerholm 1962, Uvnäs 1963) that with high concentration of compound 48/80 the release of histamine from rat peritoneal mast cells is started within 6 sec and is completed within 20 sec With the present method it was possible to follow quantitatively the course of the release during short periods From the release curve at 37°C it is evident that the process was started within less than 5 sec and nearly finished after 10 sec, i e it required 5—10 sec for completion. However, the time curve represents the sum of events in the whole cell population, and it may not reflect the release process in a single cell The significance of the time curves will be discussed in more detail in a forthcoming paper (Bloom, Fredholm and Haegermark, to be published) It is quite evident, however, that the time course had the same appearance for F II and bee venom, as well as for compound 48/80 On the other hand, the time curves have another shape after treatment of the cells with decylamine (Bloom, Fredholm and Haegermark, to be published), a substance which induces histamine release in a different way (Moran, Uvnäs and Westerholm 1962)

The results of the present investigation have thus offered strong support to the assumption that F II releases histamine from rat peritoneal mast cells by a mechanism similar to that of compound 48/80 It is interesting to note that the histamine releasing effect of melittin, a peptide isolated from bee venom (Habermann 1963) was reported by Rothschild (1965) to have little of the precise biochemical requirements shown to condition the action of Compound 48/80

The results with unfractionated bee venom were essentially the same as with F II However, there were some discrepancies Preheating the cells at 47 C for 10 min far more reduced the action of F II than that of bee venom It was also observed that high concentrations of ninhydrin (10^{-3} M) potentiated the releasing effect of bee venom, whereas the effect of F II was inhibited Thus it seems that F II is not the sole histamine releasing factor in bee venom In fact preliminary results indicate that a histamine releasing principle with somewhat diverging properties is present in the unfractionated material and eluted from the Sephadex before F II In contrast to F II this factor is hemolytic and has the ability to release 5-hydroxytryptamine from rabbit platelets (Fredholm and Westerholm to be published)

This investigation has been supported by grants from Karolinska Institutet and from Konung Gustaf V:s 80-årsfond, Sweden, and from the National Institutes of Health, U.S.A. (Grant No AM 04063-08) which are gratefully acknowledged

Compound 48/80 and bee venom were generously supplied by AB [illegible] Helsingborg, Sweden and lecithin by Dr A Wretlind, National Institute of Public Health, Stockholm, Sweden

References

BERGENDORFF, A, 1963 Personal communication

BLOOM, G D and Ö HAEGERMARK, A study on morphological changes and histamine release induced by compound 48/80 in rat peritoneal mast cells *Exp Cell Res* 1965 *40* 637—654

BRAY, R E and P P VANARSDEL, *In vitro* histamine release from rat mast cells by chemical and physical agents *Proc Soc exp Biol (N Y)* 1961 *106* 255—259

FELDBERG, W and C H KELLAWAY, Liberation of histamine from the perfused lung by snake venoms *J Physiol* (Lond) 1937a *90* 257—279

FELDBERG, W and C H KELLAWAY, Liberation of histamine from the perfused lung of the guinea pig by bee venom *J Physiol* (Lond) 1937b *91* 2P—3P

FREDHOLM, B, Studies on a mast cell degranulating factor in bee venom *Biochem Pharmacol* 1966 *15* 2037—2043

HABERMANN, E, Recent studies on *hymenoptera* venoms *Proc 2nd internat pharmacol meeting*, Prague 1963 *9* 53—62

HOGBERG B and B UVNAS, The mechanism of the disruption of mast cells produced by compound 48/80 *Acta physiol scand* 1957 *41* 345—369

HOGBERG, B and B UVNAS, Further observations on the disruption of rat mesentery mast cells caused by compound 48/80, antigen-antibody reaction, lecithinase A and decylamine *Acta physiol scand* 1960 *48* 133—145

MORAN, N C, B UVNAS and B WESTERHOLM, Release of 5 hydroxytryptamine and histamine from rat mesentery mast cells *Acta physiol scand* 1962 *56* 26—41

PATON, W D M, Histamine release by compounds of simple chemical structure *Pharmacol Rev* 1957 *9* 269—328

ROTHSCHILD, A M, Histamine release by bee venom phospholipase A and mellitin in the rat *Brit J Pharmacol* 1965 *25* 59—66

SHORE, P A, A BURKHALTER and V H COHN, Jr, A method for the fluorometric assay of histamine in tissues *J Pharmacol* 1959 *127* 182—186

SINGLETON, E M and S L CLARK, Jr, The response of mast cells to compound 48/80 studied with the electron microscope *Lab Invest* 1965 *14* 1744—1763

[illegible]

UVNAS, B and I L THON, Isolation of "biologically intact" mast cells *Exp Cell Res* 1959 *18* 512—520

UVNAS, B and I-L THON, Evidence for enzymatic histamine release from isolated rat mast cells *Exp Cell Res* 1961 *23* 45—57

UVNÄS, B, B DIAMANT and B HOGBERG, Trigger action of phospholipase A on mast cells *Arch int Pharmacodyn* 1962 *140* 577—580

UVNAS, B and J ANTONSSON, Triggering action of phosphatidase A and chymotrypsin on degranulation of rat mesentery mast cells *Biochem Pharmacol* 1963 *12* 867—873

Acta physiol. scand. 1967. 69. 313—319

From the Coagulation Research Department, Karolinska Institutet, the Medical Department and the Department of Endocrinology, Karolinska Sjukhuset, Stockholm Sweden

Coagulation Factors and Defibrination Syndrome in Anaphylaxis

By

MARGARETA BLOMBÄCK, STIG-ARNE JOHANSSON and HANS ERIK SJOBERG

Received 28 October 1966

Abstract

BLOMBACK, M, S A JOHANSSON and H-E SJOBERG *Coagulation factors and defibrination syndrome in anaphylaxis* Acta physiol scand 1967 *69* 313—319

Coagulation factors were studied in rabbits with induced anaphylaxis and in control rabbits In the anaphylactic group a decrease in platelets, coagulation factors V, IX, II+VII and fibrinogen was noted as well as an increase in antithrombic activity In the control group an increase in factor IX was noted The other factors were unchanged We believe that increased intravascular coagulation is the cause of the changes in the coagulation factors and that it might at least in part contribute to the pathophysiology of the anaphylactic syndrome Heparin treatment of conditions with consumption of coagulation factors is discussed

As heparin administration may prevent the development of anaphylactic reactions (Kyes and Strauser 1926) and also inhibits the decrease in number of platelets (Johansson 1960) it was suggested that diffuse intravascular coagulation could play a part in the development of anaphylactic reactions In the present paper the preliminary studies reported by Johansson 1960 1961a) of variations in the content of several coagulation factors and in antithrombic activity in rabbit plasma during anaphylaxis have been completed

Materials and methods

Rabb [illegible]

throughout the experiment

Collection of blood samples Blood was drawn from the catheter in the carotid artery into a liconize glass tubes containing 0 5 volume of 3 8 % trisodium citrate (+2 H_2O) per 4 5 volumes of [illegible]

TABLE I Mean value and range of different analyses in 8 sensitized anesthetized rabbits before and

	Before injection	Minutes after injection of 1 ml of 0 9 per cent saline 15	30	60
Platelets × 10[3] per μl	323(8) 280—368	320(2) 275—364	307(8) 270—340	285(6) 180—352
Coagulation time min	5 30 (8) 3 00 —7 00′	6 20 (2) 5 50′ —6 30	5 50 (8) 3 20 —8 20	5 30 (6) 4 05 —7 15
Factor V (per cent of original)	100(8)	103(2) 73—133	106(8) 73—160	114(6) 101—160
Prothrombin + Proconvertin (per cent of original)	100(8)	96(2) 96 96	100(8) 80—116	100(6) 84—108
Factor IX (per cent of original)	100(8)	187(2) 163—210	142(8) 90—173	186(6) 130—305
Fibrinogen[1] (per cent of original)	100(8)	114(2) 108—120	99(8) 92—106	104(7) 89—120
Antithrombic titre (expressed as heparin units)	0 24(8) 0 12—0 35	0 20(2) 0 18 0 21	0 23(8) 0 16—0 36	0 27(6) 0 20—0.33

[1]The original fibrinogen values ranged from 1 8 to 4 5 mg per ml Within brackets number of ana

TABLE II Mean value and range of different analyses in 7 sensitized anesthetized rabbits before

	Before injection	Minutes after injection of 1 ml of horse serum 15	30	60
Platelets × 10[3] per μl	303(7) 240—370	99(3) 90—108	123(6) 80—160	144(7 5?—184
Factor V (per cent of original	100(7)	94(3) 82—108	67(5) 37—81	57(6 22—73
Prothrombin + Proconvertin (per cent of original)	100(6)	96(3) 94—98	86(5) 69—92	74(6) 44—91
Factor IX (per cent of original	100 6	90(3) 62—107	81(5) 47—107	63((12—100
Fibrinogen* (per cent of original)	100(7	107(4) 105—112	106(6) 87—138	98(7) 90—112

*The original fibrinogen values ranged from 1 8 to 5 3 mg per ml Within brackets number of ana

23 C These samples were
ition samples were
ng Samples were
NaCl respectively

Whole blood

Hedenius (1936)

after injection of 1 ml of 0.9 % saline (controls)

120	180	240	360
287(5) 240—310	286(6) 180—350	257(2) 240, 275	297(2) 295—300
5 30 (5) 4 00′—7 30′	5 (5) 4 00′—6 00′	4 30 (2) 4 30′, 4 30	5 (2) 4 30′, 5 30′
89(5) 64—112	100(6) 65—165	134(2) 116, 152	121(2) 103, 138
99(5) 91—103	94(6) 80—115	112(2) 98, 126	92(2) 76, 108
151(5) 81—270	253(6) 124—500	189(2) 200, 168	230(2) 150, 310
100(6) 95—117	105(6) 91—125	98(6) 93—100	105(2) 98, 112
0 20(5) 0 16—0 28	0 24(6) 0 09—0 34	0 20(2) 0 20 0 20	0 17(2) 0 12, 0.21

lysed rabbits

and after injection of 1 ml of horse serum

120	180	240	300—420
126(6) 16—170	140(5) 60—236	120(2) 90, 150	85(3) 25—140
41(6) 14—74	30(6) 4—50	33(2) 26, 39	16(2) 2—30
60(6) 35—81	58(6) 19—81	59(2) 53, 65	51(2) 19—83
67(6) 34—100	56(6) 30—85	66(2) 52, 79	44(2) 28—60
91(7) 67—116	[illegible] 31—122	77(3) 69—85	72(3) 40—116

lysed rabbits

Prothrombin (factor II) and proconvertin (*factor VII*) were determined according to Owren (1951) in plasma dilutions 1:10 and 1:20

The generalized Shwartzman reaction is similar to that of anaphylaxis McKay and Shapiro (1958) showed it to be accompanied by intravascular coagulation, and Stetson (1951) noted that the hemorrhages found were due to local necrosis produced by platelet and fibrin microembolism

The role of an intravascular clotting in the etiology of shock has been pointed out in obstetric shock by Schneider (1951, 1959), in endotoxin shock by Shapiro and McKay (1958) and by Hardaway (1961), in anaphylactic shock by Johansson (1960, 1961a, 1964a), in crush injury by Bergentz and Nilsson (1961) and in burn injuries by Johansson (1961b, 1964b)

A close study of coagulation factors during manifestations of anaphylactic shock has as far as we know not earlier been performed The results of this work show, that the levels of factor V, prothrombin+proconvertin, factor IX, fibrinogen and the number of platelets decrease after injection of antigen to sensitized animals The anaphylactic shock thus is followed by decrease of most of the analyzed coagulation factors The present results are in good agreement with those reported by Johansson (1960, 1961a, 1964a) The decrease in coagulation factors and fibrinogen can most probably not be ascribed to increased fibrinolytic activity in plasma as only a very slight increase in this activity was noted The time relation between the decrease of platelets and the decrease in coagulation factors supports the idea that the decrease and disintegration of platelets release substances which can initiate the clotting of blood and is accompanied by consumption of coagulation factors We also noted the appearance of an antithrombic activity within 30 min after the eliciting shock dose was given We suggest that this increased activity is due to released heparin, as heparin has been found during anaphylaxis in dogs by Jaques and Waters (1941) and in rabbits by Reid (1950)

Heparin is known to protect animals from anaphylaxis when given before the eliciting shock dose Kyes and Strauser 1927) Johansson (1960, 1961a, 1964a, 1967) noted that heparin, when given before the shock dose prevented the decrease in platelet number, in fibrinogen content in prothrombin + proconvertin activity as well as the release of the biologically active amine, 5 hydroxytryptamine from the platelets It also prevented proteinuria and haematuria

Pretreatment with heparin has been found to have a protective action against consumption of coagulation factors in certain bleeding states in humans in obstetric shock' by Schneider (1951 1959 and Pfau, Lasch and Gunther 1960), in bleeding states such as in liver cirrhosis by Johansson (1960, cf Johansson 1964c), which latter results were confirmed by v Francken *et al* 1963) and in purpura fulminans Hjort, Rapaport and Jorgensen (1964) Protective action of heparin has also been shown in animal experiments in anaphylaxis (Johansson 1960 1961a 1964a) and burn injuries (Johansson 1961b 1964b)

The increased antithrombic activity we have found in our experiments was most probably too small or to late in most of the animals and only in rabbit nr 13 this activity seems too have been high enough to inhibit the clotting and consumption of coagulation factors

References

ACHARD Ch and M AYNAUD Les globulines dans l anaphylaxie *C R Soc Biol* (Paris) 1909 *67* 83—84

[illegible]

BLOMBÄCK B M BLOMBACK P OLSSON, G WILLIAM OLSSON and A SENNING Determination of heparin level of the blood *Acta chir scand* 1959 Suppl 245 259—264

DRAGSTEDT, C A Anaphylaxis *Physiol Rev* 1941 *21* 563—587

v FRANCKEN, I, L JOHANSSON, P Olsson and E ZETTERQVIST Heparin treatment of bleeding — clinical observations *Lancet* 1963 *I* 70—73

HEDENIUS P An apparatus for determining the coagulation time of the blood *Acta med scand* 1936 *88* 440—442

HJORT P F, S I RAPAPORT and L JORGENSEN Purpura fulminans Report of a case successfully treated with heparin and hydrocortisone Review of 50 cases from the literature *Scand J Haemat* 1964 *1* 169—192

JAQUES L B and E T WATERS The identity and origin of the anticoagulant of anaphylactic shock in the dog *J Physiol* (Lond) 1941 *99* 454—466

JOHANSSON, S A Inhibition of thrombocytopenia and 5-hydroxytryptamine release in anaphylactic shock by heparin *Acta physiol scand* 1960 *50* 95—104

JOHANSSON, S-A On the protective effect of heparin in cases of anaphylactic shock *Acta derm* [illegible]

JOHANSSON, S A Heparin and some coagulation factors in experimental burn injuries *Acta chir scand* 1964 127 346—349 (b)

JOHANSSON, S A Studies on blood coagulation factors in a case of liver cirrhosis Remission of the hemorrhagic tendency on treatment with heparin *Acta med scand* 1964 *175* 177—183 (c)

JOHANSSON S-A Intravasal koagulation vid anafylaxi *Nord Med* 1967 (in press)

KOPELOFF, N and L M KOPELOFF Blood platelets in anaphylaxis *J Immunol* 1941 *40* 471—481

KYES, P and E R STRAUSER Heparin inhibition of anaphylactic shock *J Immunol* 1926 *12* 419—422

McKAY, D G and S S SHAPIRO Alterations in the blood coagulation system induced by bacterial endotoxin I In vivo (generalized Shwartzman reaction) *J exp Med* 1958 *107* 353—367

NILSSON, I M, M BLOMBACK A THILEN and I v FRANCKEN Carriers of hemophilia A *Acta med scand* 1959 *165* 357—370

OWREN P A and K AAS The control of dicumarol therapy and the quantitative determination of prothrombin and proconvertin *Scand J clin Lab Invest* 1951 *3* 201—208

PFAU, P H G LASCH and O GUNTHER Sanarelli Shwartzman Phanomen bei febrilen Fehlgeburten und schweren Schock und Blutungs ustanden in der Geburtshilfe *Gynaecologia* (Basel) 1960 *150* 17—33

REID G Liberation of heparin and histamine by d tubocurarine *Nature* (Lond) 1950 *165* 320—321

ROCHA e SILVA, M The role played by leucocytes and platelets in anaphylactic and peptone shock *Ann N Y Acad Sci* 1950 *50* 1045—1067

SCHNEIDER, C L Fibrin embolism disseminated intravascular coagulation) with defibrination as one of end results during placenta abruptio *Surg Gynec Obstet* 1951 *92* 27—34

SCHNEIDER C L Etiology of fibrinopenia fibrination defibrination *Ann N Y Acad Sci* 1959 *75* 634—675

SHAPIRO S S and D G McKAY The prevention of the generalized Shwartzman reaction with sodium warfarin *J exp Med* 1958 *107* 377—381

[illegible]

Acta physiol. scand. 1967. 69. 320—326

From the Department of Obstetrics and Gynecology and the Department of Chemistry, Karolinska Institutet, Stockholm, Sweden

The Effect of Eight New Prostaglandins on Human Myometrium

By

M Bygdeman and M Hamberg

Received 1 November 1966

Abstract

Bygdeman, M and M Hamberg *The effect of eight new prostaglandins on human myometrium* Acta physiol scand 1967 *69* 320—326

compounds was 4—40 per cent and of the dehydrated compounds 3—30 per cent of that of the PGF-compounds

It has been shown that the prostaglandin activity of extracts of the vesicular gland of sheep (Euler 1936) is due to a group of structurally related compounds (Bergstrom *et al* 1962a, b, Samuelsson 1963a) After the structures of these compounds had been determined it was shown that the prostaglandins PGE_1, PGE_2, PGE_3, $PGF_1\alpha$ and $PGF_2\alpha$ were present in seminal plasma of man (Bergstrom and Samuelsson 1962, Samuelsson 1963b) (Fig 1)

Early work on the effect of human seminal fluid showed that it inhibited the spontaneous motility of human myometrium *in vitro* in most cases (Kurzrok and Lieb 1930, Cockrill, Miller and Kurzrok 1935) A similar effect was obtained with a crude extract of human seminal plasma (Eliasson 1959, Bygdeman 1964)

The effect of the five prostaglandins identified in human seminal plasma on human myometrium *in vitro* has also been determined (Bygdeman and Eliasson 1963a, Bygdeman 1964, Pickles and Hall 1963, Sandberg, Ingelman-Sundberg and Rydén 1963, 1964 and 1965) The three PGE-compounds (PGE_1, PGE_2 and PGE_3) inhibited the spontaneous motility in the same way as seminal fluid The sensitivity of the myometrium was most pronounced around ovulation time At this time in the menstrual cycle 0 01—0 1 μg of PGE_1 per ml bath fluid always caused a significant inhibition of the motility, PGE_2 and PGE_3 showed a similar but slightly less pro-

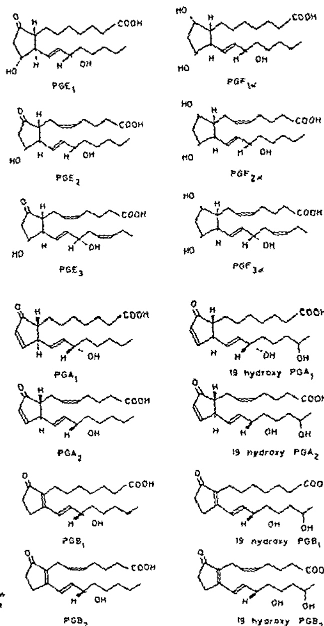


Fig 1 The structures of PGE_1, PGE_2, PGE_3, $PGF_1\alpha$, $PGF_2\alpha$ and $PGF_3\alpha$

Fig 2 The structures of the new prostaglandins identified in human seminal plasma

nounced effect Bygdeman 1964) The PGF compounds had no effect on the motility of human myometrium *in vitro* in the same dose range as the PGE-compounds Higher doses (0 3—0 5 μg/ml), however often caused a slight stimulation of the motil-

Recently eight additional prostaglandins were isolated from human seminal plasma and their structures were determined (Hamberg and Samuelsson 1965, 1966) These compounds are derivatives of the PGE compounds formed by hydroxylation at C—19 and/or dehydration involving the hydroxyl group at C—11 Four of these derivatives (PGA_1, PGA_2, PGB_1 and PGB_2) are present in about the same total concentration as the PGE compounds and the remaining four derivatives (19 hydroxy-PGA_1, 19 hydroxy-PGA_2, 19 hydroxy-PGB_1 and 19 hydroxy PGB_2*) occur in four times as high concentration (Fig 2)

The effect of the eight newly recognized prostaglandins on isolated human myometrium and rabbit jejunum is described in this report

Methods

vesicular glands and the other eight compounds from human seminal fluid according to the procedure described by Hamberg and Samuelsson (1966)

Results

The effects of the prostaglandins were recorded on 52 myometrial strips from 13 uteri All the eight compounds inhibited the spontaneous motility of the non pregnant isolated myometrium and the effect was similar to that of PGE_1 (Fig 3) In two cases out of five, however high doses (3 0 μg ml bath fluid) of 19 hydroxy-PGB_1 instead stimulated the motility

The technique used to determine the percentage activity of the prostaglandin derivates relative to PGE_1 is illustrated in Fig 4 This represents a three point determination

*These compounds were earlier referred to as PGE_1—217 PGF_1 217 PGE_2—278, PGE_1—278 19 hydroxy PGE_1—217, 19 hydroxy PGF —278 19-hydroxy PGE_2—217 and 19-hydroxy PGF_1 278 respectively The new nomenclature was introduced by Hamberg and Samuelsson 1967

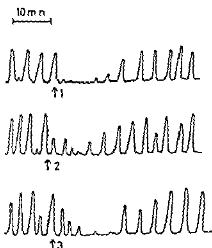


Fig 3

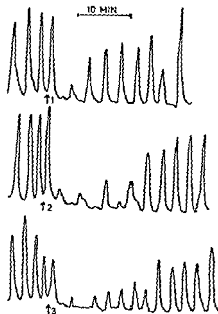


Fig 4

Fig 3 Comparison between the effect of PGE_1, 19 hydroxy PGA_1 and PGB_1 on three myometrial strips from an uterus in late proliferative phase 1=3 µg 19 hydroxy PGA_1 2=0 3 µg PGB_1, 3=0 1 µg PGE_1 All doses calculated per ml bath fluid

Fig 4 Estimation of the relationship between PGE_1 and 19 hydroxy PGB_1 on a strip of human myometrium 1=0 3 µg 19 hydroxy PGB_1 2=0 03 µg PGE_1 3=0 5 µg 19 hydroxy PGB_1 All doses calculated per ml bathfluid

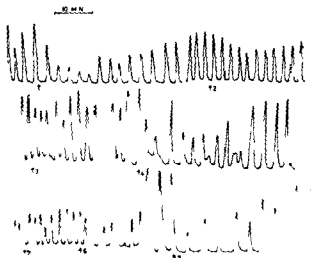


Fig 5 The additive effect of three dehydrated prostaglandins and PGE The figures shows how approximately one fourth of the ID₅₀ dose for each compound administered together gave an inhibition similar to that obtained with the ID₅₀ of each compound 1 2 µg PGB_1 2 0 8 µg PGA 3=0 3 µg PGB 4=2 µg PGB_1 5=0 01 µg PGF_1 6=0 03 µg PGE 0 01 µg PGE_1+0 2 µg PGA_1 0 5 µg PGB 0 5 µg PGB All doses calculated per ml bathfluid

TABLE I. Approximate ID_{50} doses of the eight prostaglandins and their biological activities relative to prostaglandin E_1

Substance	Human myometrium			Isolated rabbit intestine	
	ID_{50} dose µg/ml bath fluid	Per cent of activity of PGE_1	Number of uteri	Per cent of activity of PGE_1	Number of experiments
PGA_1	0.1—1.0	3—30	6	2—8	5
PGA_2	0.3—1.0	1—10	3	0.3—3	5
PGB_1	0.5—2.0	3—30	6	10—20	3
PGB_2	0.3—1.0	3—10	6	1—15	4
19 hydroxy PGA_1	1.0	2	2	1—5	2
19 hydroxy PGA_2	0.3—2.0	1—10	4	5—10	3
19 hydroxy PGB_1	*1.0—3.0*	*1—5*	5	*7—15*	*2*
19 hydroxy PGB	1.0—>3.0	<1—3	4	10	1

The results are summarized in Table I. The ID_{50} values varied somewhat with different uterine strips.

Generally the dehydrated compounds had 3—30% and the 19 hydroxylated compounds 1—10% of the inhibitory effect of PGE_1 on human myometrium.

The effects of mixtures of the compounds were also examined. In one of these experiments (Fig. 5) one fourth of the ID_{50} dose of each of PGA_1, PGB_1, PGB_2 and PGE_1 were added together and were found to have approximately the same effect as one ID_{50} dose. These results as well as similar experiments using the other derivatives indicated that the effects of tested compounds were additive.

Isolated rabbit intestine was stimulated in a similar way as PGE_1 by the 8 tested compounds. However, much higher doses had to be given to obtain an effect. PGA_1 had for instance only 7% of the smooth muscle stimulating effect of PGE_1. The results are summarized in Table I.

Discussion

All the prostaglandin compounds tested in this report inhibited the spontaneous motility of human myometrium and stimulated the activity of rabbit jejunum *in vitro* in a similar way as PGE_1. The biological activity was however, lower than that of PGE_1 on both the two test preparations. Daniels et al. (1965) tested the effect of PGA_1 on rabbit duodenum and guinea pig ileum and found that this compound had 1/100th of the smooth muscle stimulating activity of PGE_1. In our study we got a slightly higher value, two to eight per cent.

The normal effect of human seminal fluid on isolated human myometrium is an inhibition of the spontaneous motility (Kurzrok and Lieb 1930, Cockrill *et al.* 1935). If we want to evaluate the importance of the different prostaglandins for

this effect one must consider both their concentration in the seminal plasma and their effects on human myometrium

The total concentrations of the PGE compounds (PGE_1, PGE_2 and PGE_3) in semen from men with apparently normal fertility was 53 5 $\mu g/ml$ (Bygdeman and Samuelsson 1966)

Preliminary data indicated that the total concentration of the dehydrated prostaglandins was about the same as that of the PGE compounds The total inhibitory effect of the dehydrated prostaglandins is therefore about 3—30 per cent of that of the PGE compounds The 19 hydroxylated prostaglandins, present in 4 times as high concentration as the PGE compounds, should have a total effect of about 4—40 per cent of that of the PGE-compounds It is of importance, however, that the inhibitory effect of the prostaglandins present in human semen seemed to be additive From the results obtained it seems that the concentration of the newly recognized prostaglandins are of importance for the inhibitory effect of human semen on isolated human myometrium

This work has been supported by grants from the Swedish Medical Research Council (17X—753—01 and 13X—217—02, the latter to Dr B Samuelsson)

References

Bergström, S, F Dressler, R Ryhage B Samuelsson and J Sjövall, The isolation of two further prostaglandins from prostate glands *Arkiv Kemi* 1962a 19 563—567

Bergström S, R Ryhage B Samuelsson and J Sjövall, The structure of prostaglandin E_1, F_1 and F_2 *Acta chem scand* 1962b *16* 501—507

Bergström S and B Samuelsson Isolation of prostaglandin E_1 from human seminal plasma *J biol Chem* 1962 *237* PC 3005—3006

Bygdeman, M, The effect of different prostaglandins on human myometrium in vitro *Acta physiol scand* 1964 *63* Suppl 242

Bygdeman, M and R Eliasson A comparative study on the effect of different prostaglandin compounds on the motility of the isolated human myometrium *Med exp* Basel 1963a *9* 409—415

Bygdeman M and R Eliasson *The effect of prostaglandins from human seminal fluid on the* motility of the non pregnant human uterus in vitro *Acta physiol scand* 1963b *59* 43—51

Bygdeman M and B Samuelsson Quantitative determination of prostaglandins in human semen *Clin chim Acta* 1964 *10* [illegible]—[illegible]8

Bygdeman M and B Samuelsson Analyses of prostaglandins in human semen *Clin chim Acta* 1966 *13* 465—474

Cockrill, J R E C Miller Jr and R Kurzrock The substance in human seminal fluid affecting uterine muscle *Amer J Physiol* 1935 *112* 577—580

Daniel, F G J W Hinman B A Johnson F P Kupiecki J W Nelson and J F Pike The isolation of an additional prostaglandin derivative from the enzymatic cyclization of homo-linolenic acid *Biochem biophys Res Commun* 1965 *21* 413—417

Eliasson R Studies on prostaglandin Occurrence formation and biological actions *Acta physiol scand* 1959 *45* Suppl 158

Euler U S v On the specific vaso-dilating and plain muscle stimulating substances from accessory genital glands in man and certain animals prostaglandin and vesiglandin *J Physiol* (Lond) 1936 *88* 213 234

Hamberg M and B Samuelsson Isolation and structure of a new prostaglandin from human seminal plasma *Biochim Biophys Acta* 1965 *106* 215—217

Hamberg M and B Samuelsson Prostaglandins in human seminal plasma *J biol Chem* 1966 *241* 257—263

Hamberg M and B Samuelsson New groups of naturally occurring prostaglandins *I Nobel Symposium* [*on Prostaglandins*] 1967 In press

KURZROK R and C C LIEB, Biochemical structures of human semen II The action of semen on the human uterus *Proc Soc exp Biol* (NY) 1930 *28* 268—272

PICKLES, V R and W J HALL, Some physiological properties of the menstrual stimulant substances A_1 and A_2 *J Reprod Fertil* 1963 *6* 315—317

SAMUELSSON, B, The structure of prostaglandin E_3 *J Amer chem Soc* 1963a *85* 1878—1879

SAMUELSSON, B, Isolation and identification of prostaglandins from human seminal fluid *J biol chem* 1963b *238* 3229—3234

Acta physiol. scand. 1967. 69. 327—340

From the Department of Clinical Physiology, Karolinska Sjukhuset, Stockholm, Sweden

Circulatory and Respiratory Adaptation during Prolonged Exercise of Moderate Intensity in the Sitting Position

By

L.-G. Ekelund

Received 1 November 1966

Abstract

Ekelund, L.-G. *Circulatory and respiratory adaptation during prolonged exercise of moderate intensity in the sitting position.* Acta physiol. scand. 1967. 69. 327—340.

Circulatory and respiratory adaptation during prolonged exercise of moderate intensity in the sitting position were studied with heart catheterization technique in 6 healthy young men of ordinary physical fitness. The work load was chosen so that they worked at a level of 57.3 per cent of the rate of work they could perform at a pulse rate of 170 beats/min. This load gave a mean heart rate of 126.7 beats/min after 10 min and 138.5 beats/min after 60 min of work. The circulatory changes under these experimental conditions were characterized by a constant cardiac output with a continuous increase in heart rate and a continuously falling stroke volume. The systemic arterial mean blood pressure fell slightly but significantly and the mean pulmonary arterial pressure also decreased significantly from 10 to 40 min work. The total blood volume decreased 7.2 per cent from rest to 10 min work and then remained unchanged. The respiratory response was characterized by a numerical increase ($P < 0.1$) in total ventilation, dead space ventilation and an unchanged alveolar ventilation. The respiratory quotient increased from rest to the beginning of the exercise and then decreased significantly. The observed changes during prolonged exercise may be explained by a decreased vasomotor tone with a change in the compliance of the capacity vessels and the distribution of the blood volume. The relative level of work intensity seems to be more important than the body position to maintain a steady state.

In an earlier study (Ekelund 1966) the adaptation of the circulation and respiration was studied during exercise for 1 hr in supine position. Under these experimental conditions, as in a previous study of long-term exercise in the sitting position (Ekelund and Holmgren 1964) the exercise was of non-steady state type with a continuous fall in stroke volume and systemic arterial blood pressure, but with the cardiac output kept constant by an increase in heart rate. There were, however, differences in the degree of change of stroke volume and also in the type of change of pressure in the pulmonary circulation. There was also a very slight change in respiratory response in comparison with the sitting exercise.

The supine exercise was, however, performed at a lower level of relative intensity, averaging 60 per cent in supine against 77 per cent in sitting position, expressed as the actual load in per cent of the rate of work an individual could perform at a heart rate of 170 beats/min.

The present investigation was planned to allow a study to be made of the adaptation of the circulation and respiration during exercise for one hour in sitting position and at a relative work load of the same magnitude as in the *previous study in supine* position

Material

The subjects consisted of 6 healthy male volunteers aged 21—25 years All were blood donors, controlled by the Hospital Blood Bank and of ordinary physical fitness Prior to the investigation all subjects underwent clinical examination including chest X ray, ECG at rest and during exercise all of which were normal

Methods

[illegible]

technique and the methods for estimation of blood pressure, cardiac output, blood gas analysis pH and lactic acid concentration were the same as reported earlier

The *statistical calculations* were performed according to Snedecor (1959)

Procedure

Prior to the experiment a pilot test was performed to choose a load that would give the subjects a pulse rate after *10 min corresponding to the level obtained in the previous work in the supine* position Expressed in per cent of the rate of work they could perform at pulse rate 170 beats/min (W_{170}) they worked at a level of 57 3 per cent (range 54 2—61 1) or in absolute values mean 625 kpm/min (range 450—800)

The procedure for the tests proper was the same as described by Ekelund and Holmgren (1964) The room temperature was between 20—24° C and the relative humidity about 40 per cent

Results

Anthropometric data are given in detail in Table 1 *The total amount of hemoglobin* (THb) was on an average 770 g (range 560—900) corresponding to 11 0 g per kg b w (range 9 7—12 2) which is of the same order of magnitude as in previous reports from this laboratory (Ekelund 1965)

TABLE I Anthropometric data in 6 healthy young men THb = total amount of hemoglobin g W_{170} — rate of work that can be performed at a heart rate of 170 beats/min kpm min

Case, no	Age years	Height cm	Weight kg	Heart vol ml	THb g	Hb conc g 100 ml	Blood vol l	W_{170} kpm/min supine	W_{170} kpm/min sitting
1	22	178	58	650	560	12 1	5 05	800	750
2	25	170	68	880	815	14 0	6 35	1300	1350
3	21	184	78	985	810	13 1	6 80	1200	1100
4	21	189	74	720	900	14 0	7 05	1250	1300
5	23	167	69	645	770	14 6	5 80	1250	1200
6	22	187	73	760	765	12 6	6 70	900	900

The mean value for the hemoglobin concentration with the subjects in supine position was 13 4 g per 100 ml blood (range 12 1—14 6), which is of the same order of magnitude as in earlier materials from this laboratory

The total blood volume determined by the alveolar carbon monoxide method was on an average 6 29 l (range 5 05—7 05) or 89 9 ml (range 84 4—95 1) per kg b w The total blood volume determined with I^{131} albumin was on an average 6 48 l (range 5 6—7 1) After 10 min work the blood volume had decreased to an average of 6 02 l, a significant decrease of 460 ml, which is 7 1 per cent of the initial value ($P < 0\ 01$) After 50—60 min work the average blood volume was the same, 6 00 l The corresponding plasma volumes were 4 00 l at rest, 3 60 l after 10 min work, and 3 60 l after 50—60 min work

The heart volume in the prone position averaged 773 ml (range 645—985)

The rate of work that could be performed at a heart rate of 170 beats/min (W_{170}) was on an average 1 100 kpm (range 750—1 350) in the sitting position There was no significant difference between W_{170} in supine and sitting position (average 1 117 kpm/min in supine)

Circulatory response The data are presented in detail in Table II and Fig 1 and 3 *The oxygen uptake* (V_{O_2}) at rest in the supine position was + 19 5 per cent (range + 4 1—33 6) of that predicted (Harris and Benedict 1919) During exercise V_{O_2} rose after 10 min work to an average of 1 460 ml/min (range 1 190—1 740) corresponding to a mechanical efficiency of 25 5 per cent (range 22 4—30 1) During the prolonged exercise there was an almost significant increase in V_{O_2} to an average of 1 533 ml/min, corresponding to a mechanical efficiency of 23 9 per cent ($P < 0\ 05$)

The heart rate at rest was on an average 74 9 beats/min (range 63—87) After 10 min work it rose to an average of 126 7 beats/min (range 119—133) Continued work increased the heart rate of all subjects up to an average of 138 5 beats/min (range 128—153) after 60 min

The oxygen saturation of arterial blood was normal at rest (mean 97 3 per cent) and remained unchanged during the whole work period mean 96 4 per cent at the end of work)

The cardiac output at rest was on an average 8 4 l min (range 4 7—9 9) After 10 min it had increased to an average of 13 7 l min range 10 8—15 2) During the continued work the cardiac output remained unchanged mean value after 60 min 14 0 l min (range 10 2—16 2 All values at rest with the exception of case no 1 fell within the normal limits predicted from the normal regression of cardiac output on oxygen uptake Case no 1 had a high arterial venous oxygen difference and a somewhat low cardiac output in relation to the oxygen uptake During work all values fell within the normal limits in relation to oxygen uptake (Bevegård Holmgren and Jonsson 1960)

The stroke volume at rest in supine position with legs horisontal was on an average 113 7 ml (range 60—157) After 10 min sitting exercise the stroke volume was on an average 108 8 ml (range 81—127), a value which was 4 3 per cent lower (not s

TABLE II Data obtained at rest and during exercise with heart catheterization in 6 healthy young
M = mean The other symbols are the same as in Fig 1—4

Case no	Work load kpm/min	Time min	Pulse rate beats/min	$\dot{V}_{O_2}$ ml STPD/min	AVD ml/l	Q l/min	SV ml	Lactic acid meq/l
	Rest		77	240	45	5 3	69	0 82
	450	10	133	1190	110	10 8	81	1 55
	»	20	144	1220	108	11 3	79	1 60
	»	30	146	1230	121	10 1	70	1 30
	»	40	149	1170	111	10 5	70	1 60
	»	50	148	1180	110	10 8	73	1 00
	»	60	149	1220	120	10 2	68	1 05
	Rest		65	270	34	8 0	123	0 95
	800	10	119	1740	115	15 2	127	2 05
	»	20	123	1730	108	16 0	130	1 75
	»	30	124	1790	110	16 4	132	1 90
	»	40	128	1770	111	15 9	124	1 70
	»	50	127	1800	113	16 5	128	1 60
	»	60	128	1810	115	15 8	123	1 35
	Rest		63	320	39	8 2	130	0 79
	650	10	127	1630	109	15 0	118	1 90
	»	20	127	1630	108	15 1	119	1 25
	»	30	134	1680	103	16 2	121	0 90
	»	40	137	1650	108	15 3	112	0 90
	»	50	141	1680	104	16 1	114	1 00
	»	60	144	1670	104	16 2	112	1 05
	Rest		86	340	35	9 6	112	0 75
	650	10	121	1430	101	14 2	117	1 55
	»	20	124	1450	97	15 0	121	1 30
	»	30	124	1460	104	14 0	113	1 00
	»	40	126	1440	106	13 5	107	0 95
	»	50	127	1480	100	14 8	114	1 10
	»	60	128	1450	113	12 8	100	0 95
	Rest		87	320	33	9 6	111	1 00
	650	10	128	1360	100	13 5	106	1 65
	»	20	130	1520	113	13 5	104	1 45
	»	30	130	1550	103	15 0	115	1 25
	»	40	131	1560	101	15 5	118	1 35
	»	50	129	1530	108	14 1	110	1 55
	»	60	129	1550	113	13 8	107	1 65
	Rest		70	320	38	8 4	119	0 45
	550	10	132	1410	103	13 7	104	1 30
	»	20	140	1410	100	14 1	101	1 00
	»	30	136	1450	103	14 0	103	0 85
	»	40	138	1440	102	14 1	102	0 75
	»	50	144	1470	102	14 3	100	0 70
	»	60	153	1500	100	15 0	98	0 70

men RV = right ventricular PCV = pulmonary wedge pressure S = systolic D = diastolic

Pressure, mm Hg									Blood vol, l	Plasma vol, l
RV		PA			PCV	Br A				
S	D_E	S	D	M	M	S	D	M		
22	10	19	11	15	11	116	76	92	5 6	3 6
39	4	32	17	22	11	145	78	102	5 1	3 3
44	2	32	15	20	—	138	75	98		
45	1	32	12	18	—	144	77	101		
44	1	31	13	20	—	140	75	99		
45	1	33	13	21	—	138	75	98	4 9	3 1
42	1	29	12	19	10	134	73	98		
16	5	15	8	12	9	111	64	80	6 2	3 9
28	2	*24*	*10*	*16*	*9*	*107*	*64*	*88*	5 5	3 3
28	3	22	9	16	—	115	73	91		
31	2	23	9	15	—	113	70	91		
32	2	23	11	15	—	113	65	87		
34	2	22	9	15	11	112	67	84	5 7	3 5
34	2	24	9	15	—	110	64	84		
27	6	25	10	16	10	119	67	84	6 5	4 0
37	—1	34	21	27	18	154	81	107	6 2	3 7
37	—1	30	15	22	—	150	78	103		
34	—3	29	18	23	—	135	77	103		
37	—3	32	20	23	—	145	78	103		
33	—2	30	16	22	—	140	86	104	6 0	3 6
33	—3	30	18	23	—	133	77	94		
22	3	16	8	12	8	128	70	94	7 1	4 2
33	—1	31	10	17		164	79	104	6 9	4 0
32	—3	28	11	15	11	152	76	103		
33	—3	28	9	15		147	73	93		
37	—3	30	9	14		157	76	103		
35	—3	27	8	14		143	67	94	6 9	4 0
34	—4	28	9	15	12	145	76	98		
28	6	27	16	22	12	117	70	85	6 8	3 9
29	—1	26	13	20	6	139	75	99	6 0	3 3
36	—1	28	12	21		138	74	100		
34	—1	28	12	20		133	71	96		
30	—3	*28*	*13*	*19*		*132*	*70*	*89*		
32	—4	28	12	19		141	76	99	6 2	3 5
32	—4	27	15	*19*	*8*	*134*	*73*	*92*		
22	7	22	10	16	10	119	69	86	6 7	4 4
29	—3	23	7	13	9	131	71	93	6 4	4 0
33	—4	24	7	13		135	80	98		
33	—5	24	6	12		124	70	95		
33	—5	27	5	11		124	67	89		
35	—4	26	6	12		114	62	84	6 3	3 9
33	—6	24	6	12	9	123	68	91		

TABLE III Respiratory data at rest and during exercise in 6 healthy young men P_{O_2} = arterial

Case no	Work load, kpm/min	Time, min	Resp rate breaths/min	$\dot{V}_E$ l BTPS/min	$\dot{V}_{O_2}$ ml STPD/min	RQ
	Rest		14	8 3	240	0 94
	450	10	18	28 1	1190	0 85
	»	20	18	29 4	1220	0 86
	»	30	19	30 0	1230	0 85
	»	40	19	29 8	1170	0 89
	»	50	19	29 5	1180	0 85
	»	60	20	30 4	1220	0 84
	Rest		15	7 1	270	0 79
	800	10	24	41 1	1740	0 93
	»	20	26	42 3	1730	0 94
	»	30	26	43 2	1790	0 92
	»	40	25	44 3	1770	0 93
	»	50	25	44 2	1800	0 91
	»	60	25	43 4	1810	0 90
	Rest		15	8 4	320	0 80
	650	10	22	38 8	1630	0 90
	»	20	25	40 0	1630	0 88
	»	30	26	41 0	1680	0 87
	»	40	27	41 8	1650	0 89
	»	50	25	42 3	1680	0 84
	»	60	27	41 9	1670	0 85
	Rest		13	8 6	340	0 73
	650	10	21	34 6	1430	0 91
	»	20	18	34 6	1450	0 88
	»	30	17	33 4	1460	0 88
	»	40	17	34 9	1440	0 88
	»	50	17	33 6	1480	0 87
	»	60	16	32 3	1450	0 86
	Rest		13	9 2	320	0 81
	650	10	22	38 5	1360	0 96
	»	20	23	41 4	1520	0 91
	»	30	24	40 6	1550	0 89
	»	40	24	42 9	1560	0 88
	»	50	25	45 2	1530	0 92
	»	60	25	44 7	1550	0 90
	Rest		9	8 1	320	0 82
	550	10	10	30 2	1410	0 86
	»	20	10	33 3	1410	0 87
	»	30	12	34 2	1450	0 86
	»	40	11	30 4	1440	0 85
	»	50	12	34 1	1470	0 84
	»	60	13	37 6	1500	0 85

oxygen tens on mm Hg The other symbols are the same as in F g 1—4

$\dot{V}_D$ ml BTPS	Rectal temp C	Arterial				$\dot{V}_A$ l BTPS/ m n	$\dot{V}_A$ / Qc
		P_O mm Hg	P_{CO_2} mm Hg	pH un ts	Stand b carb meq/l		
203	37 1	101	39 3	7 45	—	4 9	1 0
3?4	37 6	97	41 4	7 38	—	21 4	2 0
343	37 7	98	41 0	7 40	—	22 3	2 0
329	37 8	100	39 8	7 41		22 8	2 3
3 8	37 8	98	41 1	7 41	—	22 1	2 1
431	37 8	95	42 7	7 42	—	20 4	1 9
315	37 8	99	38 6	7 39	—	23 1	2 3
155	36 7	99	43 5	7 39	24	4 2	0 5
310	37 4	97	43 7	7 35	21	3? 5	2 1
2?6	37 7	96	40 2	7 38	21	35 1	2 2
299	37 8	99	42 3	7 37	22	34 1	2 1
3?2	38 0	105	40 5	7 38	21	35 0	2 2
357	38 0	103	41 6	7 38	22	34 0	2 1
297	38 1	102	40 6	7 38	22	34 7	2 2
161	37 3	99	41 8	7 41	24	5 4	0 5
443	37 6	94	45 2	7 35	22	27 9	1 9
398	37 9	98	42 9	7 36	22	28 8	1 9
408	38 0	96	43 3	7 35	23	29 1	1 8
399	38 1	100	42 7	7 37	22	29 7	1 9
509	38 1	101	43 1	7 38	22	28 3	1 8
431	38 2	101	43 1	7 37	22	28 9	1 8
171	37 2	92	38 6	7 41	22	5 8	0 6
297	37 7	96	40 9	7 35	21	27 3	1 9
353	37 9	96	40 8	7 36	21	27 3	1 8
383	38 0	91	43 1	7 36	21	26 1	1 9
4?2	38 1	100	41 0	7 36	21	26 9	2 0
346	38 1	98	41 9	7 37	21	26 9	1 8
334	38 1	96	41 6	7 36	21	26 2	2 0
150	37 4	103	34 0	7 45	22	6 7	0 7
388	37 8	99	39 2	7 38	21	28 9	2 1
418	37 8	95	39 7	7 37	22	30 6	2 3
339	37 9	9	38 3	7 39	21	31 3	2 1
451	37 9	98	38 7	7 39	?1	30 9	2 0
4?0	37 9	99	36 3	7 40	2?	33 4	2 4
433	37 9	95	36 9	7 40	22	32 6	2 4
203	36 8	99	37 9	7 43	24	5 9	0 7
344	37 2	91	39 6	7 38	2?	26 3	1 9
383	37 2	97	37 3	7 41	2?	29 0	? 1
5?0	37 6	100	39 2	7 41	21	?7 4	? 0
337	37 8	93	40 9	7 38	23	26 1	1 9
547	37 9	94	39 8	40	?3	?6 9	1 9
615	38 1	101	38 0	7 41	23	?9 0	1 ?

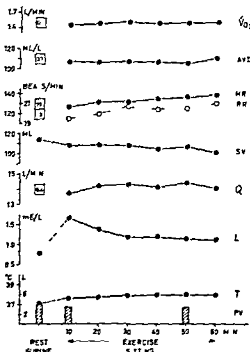


Fig 1

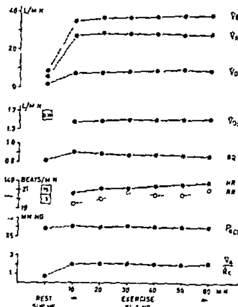


Fig 2

Fig 1 Hemodynamic data at rest, data in squares and during exercise in the sitting position for one hour mean values from 6 subjects $\dot{V}_{O_2}$ = oxygen uptake l STPD/min AVD = arterio-venous oxygen difference ml/l HR = heart rate beats min RR respiratory rate breaths min SV = = stroke volume ml, Q = cardiac output l m n,L lactic acid in arterial blood meq/l T = = rectal temperature C PV plasma volume l

Fig 2 Respiratory data at rest and during prolonged exercise in the sitting position mean values from 6 subjects $\dot{V}_E$ total ventilation l BTPS min $\dot{V}_A$ alveolar ventilation l BTPS m n $\dot{V}_D$ = dead-space ventilation l BTPS min $\dot{V}_{O_2}$ oxygen uptake l STPD m n RQ respiratory quotient, HR = heart rate beats min RR respiratory rate breaths min Pa_{CO_2} arterial carbon-dioxide tension mm Hg $\dot{V}_A$ Q_C ventilation perfusion relationship

nificantly than the resting volume Five fell below and one fell within the normal range of variation 2 S D for the relationship between stroke volume during exercise in supine position and total hemoglobin value The last was case no 2 who exercised *at the highest absolute work level In five of the subjects the stroke volume decreased* continuously during the work period with a mean of 101 3 ml (range 68—123) after 60 min for all six an almost significant change ($P < 0.05$)

Intra-cardiac and intra vascular pressures

The pulmonary wedge pressure PCV was recorded at rest in 6 after 10 min work in 5, and after 50—60 min work in 5 subjects At rest the PCV pressure was on an average 10 0 mm Hg (range 8—12 After 10 min work it was on an average 10 7 mm (range 6—18) During the continued work there were no further changes in the subjects in whom the pressure was recorded at the end of work

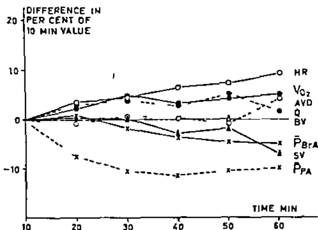


Fig 3 Hemodynamic responses during prolonged exercise in the sitting position, mean values from 6 subjects $\bar{P}_{Br_A}$ brachial arterial mean pressure, $\bar{P}_{PA}$ = pulmonary arterial mean pressure BV = total blood volume The other symbols are the same as in Fig 1 Data are presented as a percentage difference from the values obtained after 10 min work.

The systolic right ventricular pressure was at rest on an average 22 8 mm Hg (range 16—28) and rose after 10 min work to an average of 32 5 mm Hg (range 28—39) During the continued work there were no significant changes (mean value after 50 min 34 7 mm Hg) *The enddiastolic pressure in the right ventricle* was at rest on an average 6 2 mm Hg (range 3—10) and fell significantly ($P < 0\,005$) after 10 min work to an average of ± 0 mm Hg (range — 3 — + 4) and after 60 min work to — 2 3 mm Hg (range — 6 — + 1), a further decrease of 2 3 mm Hg, which was significant ($P < 0\,01$)

The systolic pulmonary arterial pressure was at rest on an average 20 7 mm Hg (range 15—27) and rose after 10 min work to 28 3 mm Hg (range 23—34) During the continued work there was no further change *The mean pressure in the pulmonary artery* was at rest on an average 15 5 mm Hg (range 12—22), 19 2 mm Hg (range 13—27) after 10 min work and 17 2 mm Hg (range 12—23) after 60 min work, a decrease of 2 0 mm Hg between 10—60 min work, which was almost significant ($P < 0\,02$)

The systolic brachial pressure was on an average 118 3 mm Hg (range 111—128) at rest, 140 0 mm Hg range 107—164) after 10 min work and 129 8 mm Hg (range 110—145) after 60 min work (mean change 11 2 mm Hg) The corresponding figures for diastolic pressure were 69 3 mm Hg (range 64—76), 74 7 mm Hg (range 64—81) after 10 min work and 71 8 mm Hg (range 64—77) after 60 min work *The average mean pressure* was at rest 86 8 mm Hg (range 80—94), 98 8 mm Hg (range 88—107) after 10 min work and 92 8 mm Hg (range 84—98) after 60 min work (mean change — 6 mm Hg) The decrease in mean pressure was almost significant ($P < 0\,02$ The pulse pressure increased from 49 0 mm Hg at rest to 65 3 mm Hg after 10 min work and then decreased to 58 0 mm Hg after 60 min work

Respiratory response The results of the respiratory studies are presented in detail in Table III and Fig 2 and 4 *The minute ventilation* (V_E) increased from an average value of 8 3 l BTPS/min (range 7 1—9 2) to 35 2 l BTPS min after 10 min w

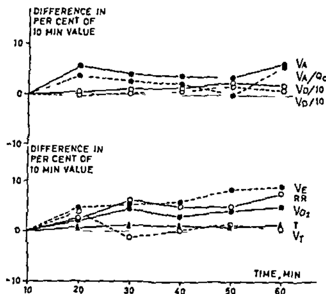


Fig 4 Respiratory responses during prolonged exercise in the sitting position, mean values from 6 subjects V_D = physiological dead space, V_T = tidal volume The other symbols are the same as in Fig 2 Data are presented as a percentage difference from the values obtained after 10 min work

(range 28 1—41 1) It thereafter increased slightly to an average of 38 4 l BTPS/min (range 30 4—44 7) after 60 min work ($P < 0.1$)

Alveolar ventilation (V_A) increased from an average value of 5 5 l BTPS/min (range 4 2—6 7) at rest to an average of 27 4 l BTPS/min (range 21 4—32 5) after 10 min work V_A increased slightly in five of six subjects in relation to the slight increase in oxygen uptake In the sixth subject the alveolar ventilation decreased very slightly V_D increased from an average of 7 8 l BTPS/min at rest (range 3 9—10 9) to 9 3 l BTPS/min (range 6 1—13 0) after 10 min ($0.1 < P < 0.2$)

The respiratory quotient, RQ, at rest averaged 0 82 (range 0 73—0 94) After 10 min work RQ had risen to 0 90 (range 0 85—0 96) It then decreased slightly to an average of 0 87 (range 0 84—0 90) after 60 min work ($P < 0.025$)

The arterial carbon dioxide tension (Pa_{CO_2}) at rest averaged 39 1 mm Hg range 34—44) During work Pa_{CO_2} remained constant, on an average 41 7 and 39 8 mm Hg after 10 and 60 min

The pH of arterial blood was on an average 7 42 (range 7 39—7 45) at rest After 10 min work there was a slight decrease to an average of 7 37 (range 7 35—7 38) and then a slight increase to an average of 7 39 (range 7 36—7 41) after 60 min

The standard bicarbonate at rest averaged 23 2 meq/l (range 22—24), after 10 min work 21 4 meq/l (range 21—22) and after 60 min work 22 0 meq l (range 21 23)

Base excess at rest averaged — 2 9 meq/l (range — 3 2—0), after 10 min work — 3 4 meq/l (range — 2 4— —4 6) and after 60 min work — 2 6 meq l range — 0 8— — 4 0)

The lactic acid concentration in arterial blood at rest averaged 0 80 meq l During exercise it rose to an average value of 1 67 meq/l after 10 min and then decreased to 1 13 meq/l after 60 min ($P < 0.01$)

The overall ventilation-perfusion relationship of the lungs ($V_A/\dot{Q}_C$) The V_A/Q_C at rest averaged 0 67 (range 0 5—1 0) After 10 min work it had risen to 1 98 (range 1 9—2 1) During continued work it showed no significant change, the value after 60 min being on an average 2 10 (range 1 8—2 3).

Rectal temperature at rest averaged 37 1° C (range 36 7—37 4) During the work period there was a continuous increase to an average of 38 0° C (range 37 8—38 2) after 60 min

Discussion

The subjects used in the present investigation were healthy young men (mean age 22 3 years) of ordinary physical fitness They had about the same anthropometric data as the subjects used in the earlier studies of cardiopulmonary response to prolonged exercise (Ekelund and Holmgren 1964, Ekelund 1966)

They worked at a medium level of intensity, on an average 57 3 per cent of the rate of work they could perform at a heart rate of 170 beats/min and with a 10 min heart rate of 126 7 beats/min The relative work level was therefore very close to that used in the investigation in supine position (Ekelund 1966) The absolute load was also of the same magnitude, mean 625 kpm/min

The mean pressure in the pulmonary artery decreased by 2 2 mm Hg (10 4 per cent) between 10 and 40 min work and then remained unchanged A decrease in the central blood volume as a result of a change in tone of the capacity vessels is one important factor which could cause such a decrease in mean pressure

In the five cases in which the pulmonary artery wedge pressure also was recorded, this remained unchanged so that the calculated pulmonary vascular resistance decreased 14 3 per cent between 10 and 60 min work The total pulmonary vascular resistance (pressure drop over the pulmonary vascular bed divided by flow consists of a network of resistances in parallel and series This resistance work in an alinear mode where one of the alinear elements are the influence of a critical closing pressure (the vascular waterfall Permutt and Riley 1963 A change in alveolar pressure can change the transmural pressure in the lung capillaries through the point of critical closing pressure and a change in the intrapulmonary pressure can act in the same way on the other pulmonary vessels Such mechanisms will probably operate mostly in the beginning of exercise when the increase in ventilation takes place

In the sitting position at rest the apical regions of the lung are less perfused than the basal regions (West 1962 Anthonisen and Milic Emili 1966 The pressure increase in the pulmonary artery during work changes the distribution of flow so that the pulmonary vascular bed is more evenly perfused Such recruitment of inactive vascular bed may result in a decrease in pulmonary arterial mean pressure without any change in left atrial pressure Such a mechanism would act rather fast Holley et al 1966 Bryan et al 1964 and is therefore one component in the total pulmonary vascular resistance which will decrease on change from rest to exercise During continuous exercise however there is likely to be a more uneven distribution of

the perfusion in relation to the beginning of the exercise due to an altered distribution of blood within the capacity vessels Such a mechanism would counteract a decrease in the pulmonary arterial mean pressure

Another factor which may change the pulmonary vascular resistance is a change in the diameters of the perfused vessels which may be rendered passive by the higher pressure during work or active due to a change in the vasomotor tone The passive dilatation of the pulmonary vessels might occur at the beginning of exercise but will probably remain unchanged during prolonged exercise Therefore a change in vasomotor tone seems to be the factor which causes the fall in pulmonary arterial mean pressure during continuous prolonged exercise This is supported by the observations of a prolonged decrease in pulmonary arterial mean pressure after an exercise period reported by Widimsky Berglund and Malmberg (1963)

The pulmonary vascular input impedance decreases when the frequency increases (Milnor Bergel and Bargainer 1966) and therefore the increase in heart rate might give a decrease in pulmonary arterial mean pressure The observed increase in heart rate counted at a percentage is however small and the initial heart rate level relatively high why such a mechanism not will be of any greater importance during work The study by Sancetta and Rakita (1957) supports that assumption because they found a decrease in pulmonary arterial mean pressure without any change in heart rate

The mean pressure in the brachial artery decreased significantly by 6 0 mm Hg or 6 1 per cent This decrease was partly accompanied by an insignificant decrease of the resistance in the systemic circulation In the report of Widimsky *et al* (1963) there was also a longstanding decrease in the brachial artery mean pressure so that most probably the cause of this decrease as well is a decrease in the vasomotor tone

The stroke volume after 10 min work was 4 3 per cent lower than the resting stroke volume which is in agreement with the findings of Bevegard *et al* (1960) When the stroke volume is measured with the subjects resting in sitting position the stroke volume is much smaller (about 40 per cent) according to earlier investigations (Bevegård *et al* 1960 Ward *et al* 1966) but with the aid of the pump function of the muscles in the legs and the abdomino thoracal pressure variations in combination with a change in tone of the capacity vessels it almost reaches the resting value in supine position During prolonged exercise the effect of the muscle pump can be assumed to be the same but the stroke volume decreases on an average by 7 9 per cent from 10 to 60 min work Therefore the cause of this decrease is probably a decrease in filling energy due to an altered distribution of blood (see Ekelund 1966) as a result among other things of a change in tone of the capacity vessels

The respiratory variables showed no significant changes with respect to minute ventilation alveolar ventilation dead space ventilation or respiratory rate But this does not exclude moderate changes in local ventilation perfusion ratio as a result of the gravitational influence and the assumed change in vascular capacity at the beginning of exercise (Ekelund and Holmgren 1965) In two earlier studies (Ekelund and Holmgren 1964 1965 there was a significant increase in total ventilation as a

result of an increased dead space ventilation The relative work intensity in both these studies was higher, however, about 75 per cent of the rate of work that could be performed at a pulse rate of 170 beats/min, so that the influence of prolonged exercise on the respiratory response seems to depend on the relative intensity of work in the main

Comparison with the study in the supine position (Ekelund, 1966)

When the study in the supine position was performed, the intention was to diminish the gravitational influence The relative working intensity, however, was lower than in the study in sitting position (Ekelund and Holmgren 1964) Therefore the present study in the sitting position was performed with lower working intensity With the aid of pilot tests almost identical experimental conditions were established with the exception of the difference in body position The respiratory reactions were almost identical and also the circulatory reaction, with the exception of the magnitude of change in pulmonary arterial pressure In the supine position there was a more marked and earlier decrease in mean pressure of the pulmonary artery, which has also been described by Sancetta and Rakita (1957) at much lower work loads The different distribution of perfusion at rest in supine and sitting positions might be of great importance for the pressure level during work in the different positions That the pulmonary vessels are under a vasomotor influence is accepted, but the role played by the sympathetic nervous system is under discussion A difference of sympathetic influence might be one factor which alters the pressure response in the sitting compared with the supine position, because the sitting or standing position strongly activates the sympathetic nervous system (Vendsalu 1960)

The other circulatory variables behave in the same way and with the same relative magnitude of change, so that posture does not seem to be the main factor affecting the ability to maintain steady state during prolonged exercise in the sitting position The results from this and earlier studies Ekelund and Holmgren 1964 1965 Ekelund 1966) imply that the relative level of intensity seems to be much more important The degree of physical fitness is also important as Holmgren 1956 reported that very well trained athletes could perform prolonged exercise in steady state in respect to heart rate) at a pulse level of 160—175 beats min

The present investigation shows that the observed hemodynamic changes in sitting occur to almost the same extent in supine when the influence of gravitation on the distribution of the blood volume within the capacity vessels has been diminished This seems to indicate the presence of a common cause of such changes during exercise in the two body positions The cause might be a diminished vasomotor tone affecting above all the capacity vessels

This study has been supported by grants from Karolinska Institute Reservationsanslaget, Stockholm and from Riksidrottsförbundets poliklinikkommittee

References

ANTHONISEN, N. R. and J. MILIC EMILI, Distribution of pulmonary perfusion in erect man. *J. appl. Physiol.* 1966. *21.* 760—766.

BEVEGÅRD, S., A. HOLMGREN and B. JONSSON, The effect of body position on the circulation at rest and during exercise, with special reference to the influence on the stroke volume. *Acta physiol. scand.* 1960. *49.* 279—298.

BRYAN, A. C., L. G. BENTIVOGLIO, F. BEEREL, A. MACLEISH, A. ZIDULKA and D. V. BATES, Factors affecting regional distribution of ventilation and perfusion in the lung. *J. appl. Physiol.* 1964. *19.* 395—402.

EKELUND, L.-G., Determination of blood volume. *Scand. J. clin. Lab. Invest.* 1965. *17.* Suppl. 86. 53—61.

EKELUND, L. G., Circulatory and respiratory adaptation during prolonged exercise in the supine position. *Acta physiol. scand.* 1966. *68.* 382—396.

[illegible] Washington. 1919. Publ. 279.

HOLLEY, H. S., A. DAWSON, A. C. BRYAN, J. MILIC EMILI and D. V. BATES, Effect of oxygen on the regional distribution of ventilation and perfusion in the lung. *Canad. J. Physiol. Pharmacol.* 1966. *44.* 89—93.

HOLMGREN, A., Circulatory changes during muscular work in man. *Scand. J. clin. Lab. Invest.* 1956. *8.* Suppl. 24.

MILNOR, W. R., D. H. BERGEL and J. D. BARGAINER, Hydraulic power associated with pulmonary blood flow and its relation to heart rate. *Circulat. Res.* 1966. *19.* 467—480.

PERMUTT, S. and P. P. RILEY, Hemodynamics of collapsible vessels with tone: the vascular waterfall. *J. appl. Physiol.* 1963. *18.* 924—932.

SANCETTA, S. M. and L. RAKITA, Response of pulmonary artery pressure and total pulmonary resistance of untrained convalescent man to prolonged mild steady state exercise. *J. clin. Invest.* 1957. *36.* 1138—1149.

SNEDECOR, G. W., *Statistical methods.* Iowa State College Press. Iowa 1959.

VENDSALU, A., Studies on adrenaline and noradrenaline in human plasma. *Acta physiol. scand.* 1960. *49.* Suppl. 173.

WARD, R. J., F. DANZIGER, J. J. BONICA, G. D. ALLEN and A. G. TOLAS, Cardiovascular effects of change of posture. *Aerospace Med.* 1966. *37.* 257—259.

WEST, J. B., Regional differences in gas exchange in the lung of erect man. *J. appl. Physiol.* 1962. *17.* 893—898.

WIDIMSKY, J., E. BERGLUND and R. MALMBERG, Effect of repeated exercise on the lesser circulation. *J. appl. Physiol.* 1963. *18.* 983—986.

Acta physiol. scand. 1967, 69, 341—347

From the Department of Physiology, University of Turku, Finland

A Mathematical Definition of the Tonicity of the Galvanic Skin Reaction in the Cat

By

A. H. Lang

Received 7 November 1966

Abstract

Lang, A. H. *A mathematical definition of the tonicity of the galvanic skin reaction in the cat.* Acta physiol. scand. 1967. *69*. 341—347.

Little attention has previously been paid to the time characteristics of the galvanic skin reaction (GSR). According to recent studies carried out in this laboratory, the intensity and time course of the GSR can be controlled differentially by the CNS. For analysing GSR dynamics quantitatively a reliable method of measuring its time characteristics is needed. The paper deals with this general problem of measurement. Based on empirical material it is suggested that the ratio of the area of the GSR curve (measured planimetrically) to the product of its peak amplitude and the recording speed is a reliable index of the tonicity of most GSRs recorded under various experimental conditions. The ton

expres

Tl is e

amplit

same way as that for the total reaction. Examples from different experiments illustrating the need and the employment of the new method of analysis are given.

The galvanic skin reaction may be defined as a transitory change either in skin resistance (exosomatic GSR) or in skin potential (endosomatic GSR) evoked by various exogenic stimuli. The endosomatic GSR involves both a faster, always negative component and a slower, mostly positive component. In the cat the negative component is normally the fairly dominating one (Lang 1967), while in man the two components exhibit variable phase and amplitude relationships (Holmquest and Edelberg 1964). In the following, unless otherwise stated, the GSR refers only to the faster, negative component of the endosomatic GSR of the cat. Also in the absence of exogenic stimuli 'spontaneous' fluctuations in skin resistance or potential may occur which may be called spontaneous galvanic skin activity. It is probable that these evoked and spontaneous electric variations, at least the negative component of the endosomatic GSR, are related to the activity of the sweat glands. In the cat the sweat glands are restricted to the hairless skin areas in the paws and are innervated by sympathetic nerve fibres (*cf.* Wang 1957).

The methods employed when using the GSR as an index of autonomic regulatory functions can, as regards their principles, be related to one of the following groups (i) comparison of the amplitudes of the GSR induced by different stimuli, (ii) study of the latencies of the reactions after stimulation, (iii) comparison of the amplitudes of the primary and the conditioned GSR produced by pairs of stimuli (Bonvallet 1960, Wang 1961), and (iv) study of the spontaneous potential waves and their degree of synchonization in different limbs (Wang 1964). Few attempts have been made to determine whether the time characteristics (shape, duration) of the GSR itself also can be used as index of autonomic regulatory functions. A reason for this may be that the duration and the shape of a GSR is harder to define and measure than for example the amplitude or the latency of the reaction. The difficulties are obvious since the curve often shows an exponential decay with random undulations.

In the cat, the endosomatic GSR evoked reflectorily by electrical tetanization of a cutaneous nerve is considered to be a phasic type of reaction showing significant variations only in amplitude (Wang and Brown 1957). According to Traxel (1957) an adequate index of the intensity of the exosomatic GSR of man can be obtained by measuring the time for decay to half maximum amplitude. The main idea in Traxel's argument is that the intensity and duration of the reactions are thought to be dependent on each other, and that the duration of the reaction will therefore indirectly reflect the intensity of the reaction. As judged by our own experience, however, the amplitude and duration of the endosomatic GSR of the cat can be affected differentially by, for example, variation of the stimulation parameters by afferent or efferent nerve tetanization, the site of stimulation in the CNS, the animal's temperature and the level of anaesthesia. The same observations in principle have been made in man. When stimulating Forel's field Spiegel et al. (1964) found that the duration of the GSR can be varied by changing the site of the stimulation. It is also known that slow changes in the level of consciousness are accompanied by prolonged changes of skin resistance whereas short phasic resistance changes are typical after momentary startling stimuli (Oswald 1962). Ebbecke who in 1931 studied the activity of sweat glands in humans by direct visual observation divided the sweat gland activity into two types: phasic and tonic.

Consequently it seems desirable to devise adequate methods of measuring not only the amplitude but also the time characteristics of the GSR. The method suggested by Traxel (1957) and the method previously used in this laboratory (Lang 1963) are similar in the way in which the time constant of an RC coupled circuit is determined. The course of action is somewhat arbitrary, however, since the irregular undulations often seen in the descending phase of the endosomatic GSR curve of the cat can cause random positional changes of the intersection with the horizontal reference line or there may be more than one intersection. Figs. 4 and 3 illustrate the irregularity in the GSR commonly obtained by tetanization of the central and afferent parts of the reflex arc. The only common features of the curves shown are (i) the abrupt beginning, (ii) the clearly distinguishable peak of the reaction located (generally the first peak) at the beginning of the curve, and (iii)

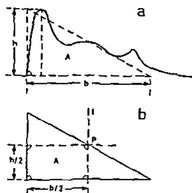


Fig 1 a b Geometrical demonstration of the principle used in the estimation and the nature of the TI
For explanation see text

the later falling tendency of the curve — Our results indicate that the features of the curve shape enumerated above are common to almost all endosomatic GSRs of the cat evoked by various stimuli in different experimental conditions (work in preparation) Corresponding observations have been made in other laboratories (Sato, Tsushima and Fujimori 1955)

The principle of the new mathematical definition

An alternative method is to integrate of the curve, i e to measure the magnitude of the area between the GSR curve and the base line Lacking physiological data on the origin of the different subphases in a GSR, all the undulations except the early peak must be considered to be random in origin This further speaks in favour of the planimetric measurement by which the whole curve is treated as an entity The planimetry can in this case also be seen as an averaging of the original curve This can be visualized by transforming the GSR curve into a right angled triangle (Fig 1) having the same area and being equal in height to the reaction curve which is transformed (side h — magnitude of the peak amplitude of the GSR) The triangle represents now an integrated and averaged GSR curve The slope of the hypotenuse is an average measure of the general slope of the GSR curve

It is also easy seen that the ratio of the area and the peak amplitude of the GSR reflects the general or average slope of the descending part of the curve (considering that the ascending part is only a relative small part of the total curve) The more gently sloping the curve the greater is the ratio of its area and peak amplitude A customary expression for physiological phenomena with a weak declining tendency is the term tonic Thus the area — peak amplitude ratio may be said to be an index for the tonicity of the GSR Of course this ratio increases in proportion to the recording speed

The tonicity or time index (TI) of the GSR The ratio of the area of the GSR (or a particular part of this area) to the product of the peak amplitude of the GSR and the recording speed is proposed as an index of the tonicity of the GSR This index will be designated as TI (tonicity or time index)

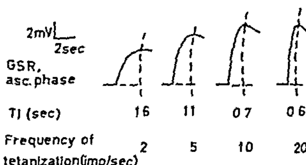


Fig 2 *Ascending phases of GRSs to efferent nerve tetanizations of different stimulus frequencies* Stimulus produced by means of a square pulse generator (Grass S-4 stimulator and isolation unit design)

were performed by means of a low level d c preamplifier (input resistance 200 kΩ) and recording unit (Grass model 5PI preamplifier Grass 5 polygraph) Succinylcholine restraint, barbiturate anaesthesia and artificial respiration were employed

Note the decrease of the TI and the shortening of the ascending phase despite a simultaneous increase in the peak amplitude as the result of increasing tetanization frequency Broken lines bound the ascending phase area measured for the calculation of the TI

The ordinate of the curve representing the GSR (Fig 1 a) gives the intensity of a biophysical effect in mV The area of the figure which is bounded by the GSR curve and the time axis shows the time integral of this effect This area measured planimetrically is denoted by the symbol A The maximum amplitude of the GSR curve is denoted by the symbol h If, during recording of the GSR, the amplifier is set so that 1 cm on the intensity axis corresponds to k mV, and the speed of the recording paper is such that p mm on the time axis corresponds to *1 sec 1 cm corresponds to 10 p sec* the area 1 cm² = k mV 10/p sec

$$\text{TI} \quad \frac{A \ k \ mV \ 10 \ p \ sec}{h \ k \ mV} - 10 \ A/h \ p \ sec$$

Example A = 6 0 cm² h − 2 5 cm p = 3 mm/sec
TI = 10 6 0 2 5 3 − 8 0 sec

It can be seen that the TI is merely a length of time The amplification constant and the mVs cancel This means that in an estimation of the TI the absolute intensity of the GSR and the amplification factor can be disregarded All that needs be known is the peak amplitude in centimetres the area in square centimetres, and the speed of the recording paper The value of the TI of a given reaction is not, however, dependent on the speed at which the recording is carried out If for instance, the same GSR is recorded at two speeds p and 2 p *the corresponding areas of the reactions are* A and 2 A *and the value of the TI estimated from records at different speeds will thus not change see formula A recording speed best suited for the measurement of the reaction can be chosen*

The nature of the TI can be demonstrated as follows *Fig 1 b) Let us say that the lengths of the two smaller sides of the averaged GSR curve i e the right angled*



[illegible]

horizontal bars) of [illegible] were performed [illegible]

vertical lines

Note the shortening of the descending phase without a marked drop in the peak amplitude of the GSR after deepening of the anaesthesia Only a transient drop in blood pressure level is noted The changes in the corresponding TI values give a quantitative picture of the changes in the shape of the GSRs

The small undulation in the first GSR is due to a low tetanization frequency (deficient fusion).

triangle, are h cm and b cm, its area (equal to the area of the original GSR) A cm² On the horizontal axis 1 cm corresponds to 10/p sec

$A = h \cdot b/2$ cm², $b/2 = A/h$ cm, this corresponds to 10 A/h p sec = TI of the original reaction But the vertical line (1) that halves the side b cuts the hypotenuse at the point P where the 'averaged' GSR curve has fallen to 50 per cent of its peak value In other words, *the TI is equal to the time for the decay of the 'integrated and averaged' GSR curve to half its maximum amplitude*

It must be borne in mind that independence of the TI on the absolute intensity of the GSR (in mV) is only a formal consequence of the mathematical equation determining the TI Factors which increase the amplitude of the GSR will also cause a change in the TI unless the area below the GSR curve happens to increase in the same proportion as the amplitude It must also be realized that potential changes may occur during the course of a GSR which are not directly related to sweat gland activity Such distortions may be large enough to change the total area of the GSR significantly They can be caused by slow d c fluctuations of the basal potential of the skin (Lang 1967) Consequently, the TI can hardly be used as a reliable index for 'tonicity of sweat gland activity unless attention is paid also to the amplitude of the reaction and precautions are taken to exclude secondary potential variations In humans, the endosomatic GSR seems to be the sum of two potential compo of opposite polarity and different time constants (Holmquist and Edelberg The method described above can therefore hardly be employed to me GSR curves of humans

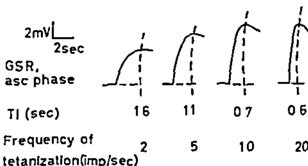


Fig 2 *Ascending phases of GRSs to efferent nerve tetanizations of different stimulus frequencies* Stimulus produced by means of a square pulse generator (Grass S 4 stimulator and isolation unit design) applied to distal stump of sectioned sciatic nerve through bipolar Ag AgCl electrodes Only the frequency changed in successive stimulations Recording of the galvanic skin activity was performed from the central pad of the corresponding hind limb with Zn $ZnSO_4$ surface electrode discs with the reference electrode at a severed dorsal surface of the same limb Amplification and record ng were performed by means of a low level d c preamplifier (input resistance 200 kΩ) and recording unit (Grass model 5PI preamplifier, Grass 5 polygraph) Succinylcholine restraint barbiturate anaesthesia and artificial respiration were employed

Note the decrease of the TI and the shortening of the ascending phase despite a simultaneous increase in the peak amplitude as the result of increasing tetanization frequency Broken lines bound the ascending phase area measured for the calculation of the TI

The ordinate of the curve representing the GSR (Fig 1 a) gives the intensity of a biophysical effect in mV The area of the figure which is bounded by the GSR curve and the time axis shows the time integral of this effect This area measured planimetrically, is denoted by the symbol A The maximum amplitude of the GSR curve is denoted by the symbol h If, during recording of the GSR, the amplifier is set so that 1 cm on the intensity axis corresponds to k mV, and the speed of the recording paper is such that p mm on the time axis corresponds to 1 sec (1 cm corresponds to 10/p sec), the area 1 cm² = k mV 10/p sec

$$TI = \frac{A \; k \; mV \; 10/p \; sec}{h \; k \; mV} = 10 \; A/h \; p \; sec$$

Example A = 6 0 cm² h = 2 5 cm p = 3 mm/sec
TI = 10 6 0/2 5 3 = 8 0 sec

It can be seen that the TI is merely a length of time The amplification constant k and the mVs cancel This means that in an estimation of the TI, the absolute intensity of the GSR and the amplification factor can be disregarded All that needs be known is the peak amplitude in centimetres, the area in square centimetres and the speed of the recording paper The value of the TI of a given reaction is not however, dependent on the speed at which the recording is carried out If, for instance, the same GSR is recorded at two speeds p and 2 p the corresponding areas of the reactions are A and 2 A and the value of the TI estimated from records at different speeds will thus not change (see formula) A recording speed best suited for the measurement of the reaction can be chosen

The nature of the TI can be demonstrated as follows (Fig 1 b) Let us say that the lengths of the two smaller sides of the averaged GSR curve, i e the right angled

reactions are seen in this phase The tonicity of the GSR in response to afferent nerve stimulation decreases with increasing depth of anaesthesia (Fig 3) — Small differences in the sites of central stimuli lead to marked differences in the shapes of the ascending and descending phases of GSRs (Fig 4)

In many cases, however, GSRs can be produced with such a small slope that the potential deviation has not yet returned to the basal level at the moment the tetanization stops For the sake of comparison, a limited time standard for the duration of stimulation in such cases must be used Two types of post tetanic phases are of special interest as regards the GSR dynamics They are the "off effect" often observed after short tetanizations in the brain stem and the GSRs of long duration coinciding with afterdischarge in amygdala (Lang, Tuovinen and Valleala 1964)

It is easy to separate different successive phases of the GSR curve on the basis of the location of its peak and the end point of, for example, the tetanization train In the latter case the latency of the reaction must be taken into consideration in order to judge where the post tetanic phase starts The quantitative analysis of this phase is a separate problem

References

BLOCH V and M BONVALLET, Le controle inhibiteur bulbaire des responses électrodermales *C R Soc Biol* (Paris) 1960 *154* 42—45

EBBECKE U Arbeitsweise der Schweissdrussen und sudomotorische Reflexe bei unmittelbarer Beobachtung mit Lupenvergrosserung *Pflugers Arch ges Physiol* 1951 *253* 222—350

HOLMQUEST, D and R EDELBERG Problems in the analysis of the endosomatic galvanic skin response *Psychophysiology* 1964 *1* 48—54

LANG H Temporal aspects of the galvanic skin response (GSR) *Acta physiol scand* 1963 *69* 86

LANG A H, Skin d c potentials and the endosomatic galvanic skin reaction *Acta physiol scand* 1967 *69* 230—241

LANG H T T [illegible]

O[illegible]

S[illegible]

SPIEGEL E A, H T WYCIS E G SZEKELY L SOLOFF J ADAMS P H GILDENBERG and C H Z[illegible] the human brain *Electro-* [illegible]

WANG G H, Poststimulatory inhibition of galvanic skin reflex in an anesthetized cat *Amer J Physiol* 1961 *200* 201—207

WANG G H *The neural control of sweating* Madison The University of Wisconsin press 1964 pp 199

WANG G H and V BROWN Terminal rebound of galvanic skin reflex in anaesthetized cats *J Neurophysiol* 1957 *20* 340—347

Acta physiol. scand. 1967. 69. 348—354

From the Departments of Zoophysiology and Pharmacology, University of Goteborg Sweden

The Series Elasticity of Active Taenia Coli in Vitro

By

A K Gunnar Åberg

Received 11 November 1966

Abstract

Åberg, A K G *The series elasticity of active taenia coli in vitro* Acta physiol scand 1967 69 348—354

The series elastic components (SEC) of the smooth muscle taenia coli of the guinea pig have been investigated [illegible] traction was approximately 30 per cent of the maximum work that the muscle could perform during an isotonic shortening

According to Hill (1938) skeletal muscle might be considered to be a two component system with active contractile elements (CE) and passive series elastic components (SEC) It was shown by Wilkie (1956) that the CE function separately from the SEC, but it is far from clear if the two components are anatomically separated (Ramsey and Street 1940, Buchthal and Rosenfalck 1960) Hill (1949) has also described parallell elastic components (PEC) which he assumed to account for resting tension of inactive muscles The aim of this work was to investigate the series elasticity of a smooth muscle and to obtain numerical values of the isometric work performed by the CE during isometric contractions

Explanation of terms

L_0 = the greatest length of an inactive muscle when the force applied to straighten it did not exceed 50 dynes (Åberg and Axelsson 1965

CE = contractile elements

SEC = series elastic components

PEC = parallell elastic components

Isometric work" denotes work performed by the CE in stretching SEC and is equal to the force times the SEC extension

"Maximum isometric work" is the isometric work performed at L_o when the *CE* exert maximum isometric force

"Isotonic work" or "physical work" is the total energy released during the shortening of a preloaded muscle Thus the work done in stretching the SEC is not included

"Maximum isotonic work" or maximal physical work" is the isotonic work performed at L_o under a load wich is 1/3 of the greatest load (P_o) which the muscle can lift. (Åberg and Axelsson 1965)

Total work equals the sum of the isometric and isotonic work.

P_o = the maximum isometric tension (in dyn) at L_o

P_1 = the isometric tension (in dyn) before release of the electromagnet (see Methods and Fig 1)

P_2 = the tension in the muscle after release of the electromagnet

Δl = extension (in mm) of SEC corresponding to the difference between P_1 and P_2

c = extension constant

Methods

Pieces of taenia coli were

by stunning and bleedin

These hooks and the m

experiments were started the muscles were contracted several times in order to avoid stair-case effects on the tension The experiments were performed in an apparatus that enabled both isotonic and isometric mea

of stainless steel

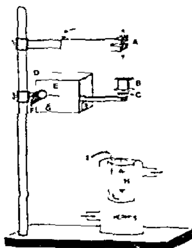


Fig 1 A Isometric transducer Grass FT 03
B Electromagnet
C. Iron plate attached to the lever
D Photoelectric transducer
E Black screen behind which a lamp is mounted in fix position
F Photoelement
G Constant load
H Muscle
I Gas mixture

the load extension curve

Results

When the isometrically contracting muscle was released and the isotonic shortening was measured the curve showed two distinct phases (Fig 2) First there was a rapid shortening, which was compleated in less than 0 1 sec, and then there was a slower phase which was not compleated until after 5—15 sec The first phase was assumed to depend on sudden shortening of SEC while the second phase depended on active shortening of the CE in the muscle Although the first phase lasted for only 0 05—0 1 sec there was supposed to have been some active shortening during that time In order to correct for this, the curve of active shortening was extrapolated till it cut a

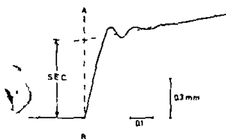
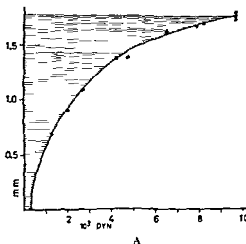
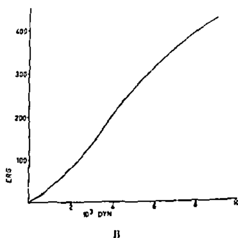


Fig 2 Isotonic shortening after release of an isometrically contracting taenia coli L_0 = 12 mm Isometric tension before release = 3500 dynes Isotonic load on the muscle after release = 1200 dynes For further description see text



A



B

Fig 3A Load-extension curve of the SEC of a taenia coli muscle The shaded area represents isometric work L_0 = 12 mm

Fig 3B Isometric work performed by the CE of a taenia during isometric contractions L_0 = 12 mm P_0 = 10 000 dynes
Max physical work = 1570 erg

vertical line (Fig 2 A—B) that had been drawn through the point where the sudden shortening had begun The distance between this latter point and the point of intersection between the extrapolated curve and the vertical line was assumed to represent real sudden shortening of extended SEC The assumption upon which this calculation is based will be discussed later

Fig 3A is a load-extension curve for the SEC and was obtained by plotting the sudden shortenings obtained after releases of an isometrically contracting muscle against the corresponding isometric tensions in the muscle before the release It is evident from the figure that the extension of SEC in taenia coli during isometric contractions was a nonlinear function of tension and in the investigated range it followed an exponential pathway The experimental data were fitted with an equation of the form

$$\Delta l = c \ \ln \frac{P_1}{P_2}$$

When the tension was 250 dynes the extension of the SEC was close to zero, and for the sake of simplicity this point on the abscissa has been taken as zero (Fig 3A)

During maximum isometric tension ($\sim 1.8 \cdot 10^6$ dyn/cm²) the SEC in the muscle was found to be stretched 13.1 ± 2.7 per cent of L_0 The isometric work performed by the CE during a contraction was equal to the force times the extension of the SEC Since the extension of the SEC was a nonlinear function of the load (Fig 3A) that work could not be measured directly (Sonnenblick 1964) but was represented by the area between the ordinate and the load extension curve of the SEC (Gasser and Hill 1924) That area (shaded in fig 3A) was calculated by means of a planimeter and was plotted against tension (Fig 3B) The maximum isometric work was found to be approximately 30 per cent (varying in 13 expts between 23 and 34 per cent) of the maximum isotonic work that the muscle could perform From Fig 3B it is evident that the isometric work developed at P_0 3 is approximately 160 erg That is about 10 per cent of the maximum isotonic work In order to calculate the total work it is necessary to add this isometric work to the physical work Fig 4) In a few muscles the

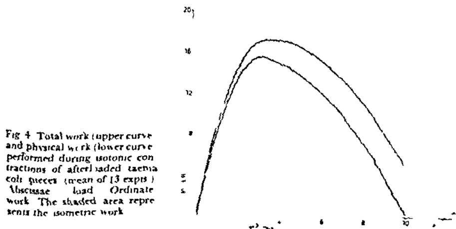


Fig 4 Total work (upper curve and physical work (lower curve performed during isotonic contractions of afterloaded taenia coli pieces (mean of 13 expts) Abscissae load Ordinate work The shaded area represents the isometric work

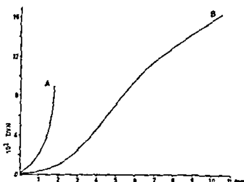


Fig 5 Comparison between the stiffness of the series elasticity of the muscle (A) and the stiffness of the whole muscle (B) Abscissae extension Ordinate tension L_0 = 12 mm Curve A was obtained in the same way as the load extension curve in Fig 3 A Curve B was obtained when the muscle was continuously stretched 4 mm/sec

stiffness of the SEC tended to decrease after some contractions (*i e* the amplitude of the sudden shortening became greater than initially) In those muscles the rate of tension development and P_0 decreased at the same time This was supposed to depend on injury or deterioration of contractile elements that thereby became inexcitable and acted like added compliances

In order to compare the stiffness of the SEC with that of the whole tissue both resting and spontaneously active muscles were stretched continuously until they broke (Fig 5) The velocity of extension was 30 per cent of L_0/sec The stiffness of the SEC was always considerably higher than that of the whole muscle

Discussion

The method that has been used to determine series elasticity in this work has some evident limitations No measurements would have been possible if the muscles had been released to zero tension As already pointed out the minimum load used in this investigation was 250 dynes The extension of the SEC at that load was called *zero* although that was an approximation making all values of extensions of the SEC a little too small

When muscles were released the load on the contractile elements suddenly decreased and their velocity of shortening was increased according to the force velocity curve It is of that reason not fully correct to extrapolate the curve of isotonic shortening linearly (Fig 2) If a muscle was released when it developed full tension the velocity of shortening had been zero at the moment of release It would then have accelerated to a velocity of shortening corresponding to the new load For striated and heart muscles with a high velocity of isotonic shortening this source of error might have been of some importance but for slowly shortening smooth muscles the error was much smaller although it lessened the real value of the extension of the SEC somewhat

The force-extension relationship of the SEC in taenia coli did not follow Hooke's law but the investigated range of the curve had an exponential shape The same has been shown for the SEC of striated muscle Aubert 1955) and heart muscle (Sonnenblick 1964)

It is difficult to make conclusions from a comparison between published observa-

tions of the SEC in different muscle types (Table I) The investigations have been performed at different temperatures and the series compliances have been shown to be greater at higher temperature (Jewell and Wilkie 1958)

TABLE I

Muscle	Elongation of SEC at P_o in per cent of L_o	Maximum tension dyn cm²	Temperature °C	References
Frog sartorius	4—9	3 5 10^{6}*	0	Katz 1939
Frog sartorius	3—4	3 5 10^{6}*	0	Hill 1950 1953
Frog sartorius	about 3	3 5 10^{6}*	0	Jewell and Wilkie 1958
Cat papillary	8—10	0 6 10^{6}	23	Sonnenblick 1964
Cat tenuissimus	9—12 8	1 4 10^{6}	37	McCrory *et al* 1966
Bovine mesenteric artery	15—20	2 1 10^{6}	37	Lundholm and Mohme Lundholm 1967
Taenia coli	12—15	1 8 10^{6}	37	

*according to Ramsey and Street 1940

Åberg and Axelsson (1965) found that the maximum physical work of afterloaded taeniae coli was performed when the load was approximately $P_o/3$ They found the maximum physical work to vary between $P_oL_o/7$ 0 and $P_oL_o/8$ 2 In this investigation the isometric work developed at $P_o/3$ was found to be approximately 10 per cent of the maximum physical work The total work performed by a taenia coli muscle that is afterloaded with $P_o/3$ is thus between P_oL_o 6 4 and P_oL_o 7 5 These values are lower than Hill s value from experiments on striated frog muscle and Csapo s (1954) value for rabbit uterus which were both P_oL_o 6

It has been suggested that the correct localization of the series elastic components of active muscles in vitro would be weak or not fully active muscle cells Blix 1895, Fischer 1926, Katz 1939 Hill 1953) In taenia coli however the stiffness of neither resting nor active whole muscles was ever as high as the stiffness of the SEC of active muscle in vitro When muscle pieces were continously stretched with a velocity of 0 3 L_o/sec, which is equal to the maximum velocity of shortening Åberg and Axelsson 1965) the stiffness of the whole tissue was never as high as that of the SEC Fig 5) Thus the stiffness of active taenia coli was not uniform in the muscle but there were weaker parts with lower stiffness than that of SEC Some structural differentiation between the SEC and other components of the muscle seems of that reason not to be impossible Edman 1966 has recently shown that the rigidity of the SEC in isolated semitendinosus fibres of the frog is independent of the state of activity of the CE This points to the fact that there is a structural differentiation between SEC and CE in striated muscles too However until the exact localization of the SEC in the muscles is known we can only state that the muscles act like models with elastic components in series with contractile elements Thus the term »sec in the muscle» must not be taken literally, but represents the properties of the muscle that are corresponding to properties of the SEC in the model

I wish to thank Associate Professor Lennart Lundholm for stimulating discussions and various help throughout this work Associate Professor K A P Edman and Professor Johann Axelsson for valuable criticism, Ing Andras Beviz for construction of the isotonic transducer and Miss Lisbeth Olsson for technical assistance

The work was supported by the Science Faculty of the University of Goteborg and AB Bofors Molndal Sweden

References

ÅBERG A K G and J AXELSSON, Some mechanical aspects of an intestinal smooth muscle *Acta physiol scand* 1965 *64* 15—27

AUBERT, X Intervention d un élément élastique pur dans la contraction du muscle strié *Arch intern Physiol Biochim* 1955 *63* 197—202

BLIX, M Zur Frage Wann der Energieumsatz bei der Muskelkontraktion auch von der Spannung abhangt *Skand Arch Physiol* 1895 *6* 245—251

BUCHTHAL, F and P ROSENFALCK Elastic properties of striated muscle Tissue Elasticity Ed by J W Remington *Amer Physiol Soc* 1957

BUCHTHAL F and P ROSENFALCK Dynamic elasticity in the initial phase of an isotonic twitch *Acta physiol scand* 1960 *49* 198—210

CSAPO, A , Dependence of isometric tension and isotonic shortening of uterine muscle on temperature and on strength of stimulation *Amer J Physiol* 1954 *177* 348—354

EDMAN K A P Contribution to a discussion *Symposium on Muscle* Budapest 1966 In the press

FISCHER E Die Zerlegung der Muskelzuckung in Teilfunktionen III *Pflugers Arch ges Physiol* 1926 *213* 352—369

GASSER H S and A V HILL, The dynamics of muscular contraction *Proc roy Soc B* 1924 *96* 398—437

HILL A V The heat of shortening and the dynamic constants of muscle *Proc roy Soc B* 1938 *126* 136—195

HILL, A V The abrupt transition from rest to activity in muscle *Proc roy Soc B* 1949 *136* 399—420

HILL, A V The series elastic component of muscle *Proc roy Soc B* 1950 *137* 273—280

HILL A V The mechanics of active muscle *Proc roy Soc B* 1953 *141* 104—117

JEWELL B R and D R WILKIE An analysis of the mechanical components in frogs striated muscle *J Physiol (Lond)* 1958 *143* 515—540

KATZ B The relation between force and speed in muscular contraction *J Physiol (Lond* 1939 *96* 45—64

LUNDHOLM L and E MOHME LUNDHOLM Relaxed length length tension diagram active stat and tone in vascular smooth muscle To be publ

MCCROREY H L GALE H H and N R ALPERT Mechanical properties of cat tenuissimus muscle *Amer J Physiol* 1966 *210* 114—120

RAMSEY R W and S F STREET Isometric length tension diagram of isolated muscle fibres of the frog *J cell comp Physiol* 1940 *15* 11 34

SONNENBLICK E H Series elastic and contractile elements in heart muscle Changes in muscle length *Amer J Physiol* 1964 *207* 1330—1338

WILKIE D R Measurements of the series elastic component at various times during a single muscle twitch *J Physiol (Lond)* 1965 *134* 527—530

Acta physiol scand. 1967. 69. 355—361

From the Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden

Cholinesterase Activity in Innervated and Denervated Sympathetic Ganglion Cells of the Cat

By

EZIO GIACOBINI, BO PALMBORG and FOLKE SJÖQVIST

Received 11 November 1966

Abstract

GIACOBINI, E., B. PALMBORG and F. SJÖQVIST. *Cholinesterase activity in innervated and denervated sympathetic ganglion cells of the cat.* Acta physiol. scand. 1967. *69.* 355—361.

Total cholinesterase [ChE, acetylcholine (AcCh) as substrate] and acetylcholinesterase [AcChE, acetyl-β-methylcholine (MeCh) as substrate] were determined with the Cartesian diver in single sympathetic cell bodies isolated from the normal and preganglionically denervated 7th lumbar ganglion of the cat. Thirty normal cell bodies showed a wide variation in total ChE activity from 3×10^{-4} to 160×10^{-4} µl CO_2/hour. Cell bodies with low activity represented the majority of the population, while cell bodies with very high activity were rare. AcChE-activity in 10 normal cell bodies varied between 3.8 and 54.4×10^{-4} µl CO_2/hr. After denervation there was a significant decrease in average enzyme activity as measured with both substrates. This is probably explained by the loss of presynaptic AcChE-rich terminals which stick to the normal cell bodies. With AcCh the enzyme activity varied between 2.7 and 28.5×10^{-4} µl CO_2/hour (n = 16) and with MeCh between 2.9 and 31.1×10^{-4} µl CO_2/hour (n = 10). Thirty-five per cent of the denervated cells (9/26) lacked measurable activity. The ratio of AcCh/MeCh hydrolysis decreased after denervation from 1.7 to 1.0. These findings support the previous concept of two functionally different cell populations in sympathetic ganglia.

Although the majority of sympathetic ganglion cells of several species contain only small amounts of AcChE[1], which is detectable by either histochemical or biochemical methods, a few cells display very high AcChE-activity (Koelle 1951, 1955, Giacobini 1956, 1957, Holmstedt and Sjöqvist 1959, Fredricsson and Sjöqvist 1962). These latter neurons are said to be cholinergic although physiological evidence for this view is available only for the "AcChE-rich" sympathetic ganglion cells of the cat. In this species, cell bodies with a characteristically intense histochemical staining for AcChE (Fig. 1 a) give rise to sweat secretory fibres (Sjöqvist 1962, 1963 a, b), which are believed to store and release acetylcholine (Dale and Feldberg 19

[1] Abbreviations used: AcChE = acetylcholinesterase
BuChE = butyrylcholinesterase
ChE = total cholinesterase

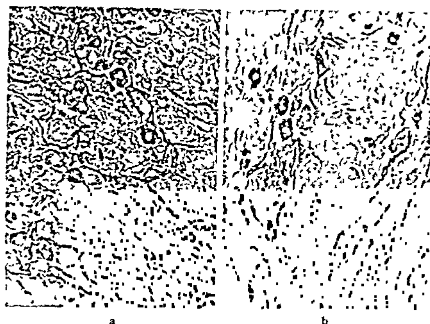


Fig 1 Seventh lumbar ganglion of the cat stained for AcChE Cryostat sections Magnification 130 × a) normal ganglion with AcChE in a few cell bodies and in surrounding fibres b) denervated ganglion with AcChE in cell bodies and in a few fibres The ganglion appears empty of stained fibres

Most sympathetic ganglion cells of the cat reveal histochemically very low or no AcChE-activity at all and have therefore been assumed to be functionally adrenergic (Koelle 1955, Fredricsson and Sjöqvist 1962, Sjoqvist 1962) In strong support of this view, these nerve cells were subsequently found to exhibit fluorescence for noradrenaline (NA) in contrast to the "AcChE-rich" cell bodies (Hamberger, Norberg and Sjöqvist 1965)

These data emphasize a quantitative difference in AcChE activity between cholinergic and adrenergic[1] sympathetic neurons The aim of this investigation was to further elucidate this difference by direct measurements of cholinesterase activity in isolated sympathetic cell bodies using a method described previously (Zajicek and Zeuthen 1956, Giacobini 1957)

The experiments were performed on cell bodies including the initial segment of the axon (approximately 50 μ long) dissected from the innervated and denervated 7th lumbar ganglion of the cat in which cholinergic, as well as adrenergic, nerve cells are known to occur (Sjoqvist 1962, 1963 b)

Furthermore, in this ganglion the proportion between presumed cholinergic and adrenergic ganglion cells has been studied with combined histochemical and pharmacological methods Sjoqvist 1962, 1963 b, Hamberger, Norberg and Sjoqvist 1965)

[1] = The term cholinergic refers to neurons giving rise to nerve terminals which are believed to have acetylcholine as the only transmitter The term adrenergic is used for neurons which release noradrenaline peripherally

Experimental

Dissection and Denervation Procedures

Twenty nine (29) adult cats of both sexes were used for the ChE determinations in normal sympathetic cell bodies dissected from the 7th lumbar ganglion

Preganglionic denervation of the 7th lumbar ganglia was performed by cutting the sympathetic chain bilaterally immediately below L6 The two denervated ganglia (L7) were dissected out three weeks later Histochemical controls (Fig 1 b) revealed that the AcChE had largely disappeared from the preganglionic nerve fibres and nerve terminals while the AcChE was still present in the sympathetic cell bodies (cf Holmstedt, Lundgren and Sjoqvist 1963)

ChE Determinations

The dissected ganglia were placed in a Ringer bicarbonate solution containing the substrate Individual cell bodies were isolated and the ChE activity determined by a modified Cartesian diver technique (Zajicek and Zeuthen 1956, Giacobini 1957), which permits quantitative determinations in the order of 1×10^{-4} μl CO_2 per hour with 5 % accuracy During the experiments the temperature of the water bath was maintained at 23 $\pm$ 0 002° C The divers had an average gas volume of 0 5 μl and an average weight of 1 mg The diver micropipette was used for sucking up the cell bodies The substrate bicarbonate solution and a small quantity of 5 % CO_2/95 % N_2 were first introduced into the diver according to Zajicek and Zeuthen (1956)

Acetylcholine iodide (AcCh final conc 5 31 $\times 10^{-3}$M) and acetyl β methyl choline (MeCh final conc 10^{-2}M) were used as substrates The incubation medium (pH 7 4) consisted of either 14 5 mg AcCh or 28 8 mg MeCh dissolved in 10 ml of a standard solution This was prepared by adding 9 6 ml of 0 85 % NaCl to 10 ml 0 20 % $NaHCO_3$ and 0 4 ml 1 19 % $CaCl_2 2H_2O$ and was saturated with a 5 % CO_2/95 % N_2 gas mixture (Giacobini 1957)

Results

The material consists of 40 normal cell bodies and 26 cell bodies from denervated sympathetic ganglia (Table I)

The total ChE activity (AcCh as substrate) of normal cell bodies shows a wide variation from 3 $\times$ 10^{-4} to 160 $\times$ 10^{-4} μl CO_2 hour (n = 30) Cell bodies with low activity represent the majority of the population while those with very high activity are rare cf Table I When MeCh was used as substrate the cell bodies showed lower enzyme activity than with AcCh (range 3 8—54 4 $\times$ 10^{-4} μl CO_2/hour, n = 10) Two out of 10 cell bodies showed markedly higher AcChE activity than the remainder In these experiments only 1 of the 40 cells lacked measurable activity (compare below

Sixteen cell denervated bodies were studied with AcCh ChE) and 10 with (AcChE as substrate Denervation caused a significant decrease in aver activity as measured with both substrates ($p < 0.01$) Thirty five per

TABLE I ChE and AcChE activities (in $\mu l\ CO_2 \times 10^{-4}$/hour) in normal and denervated sympathetic ganglion cells of the cat's 7th lumbar ganglion

Normal ganglion cells			Denervated ganglion cells	
AcCh	AcCh	MeCh	AcCh	MeCh
—	16 1	3 8	—	—
3 2	18 1	5 3	—	—
4 8	20 1	8 4	—	—
5 7	21 9	8 8	—	2 9
6 2	21 9	10 0	—	3 6
6 4	27 0	10 1	—	4 2
6 8	30 5	10 2	2 7	6 6
7 4	30 7	10 4	2 7	7 8
9 1	37 0	*47 8*	3 6	*17 1*
9 2	41 8	*54 4*	4 0	*31 1*
9 3	51 3		9 2	
12 0	73 0		10 6	
13 1	76 0		12 7	
13 7	101 0		19 5	
14 8	160 0		22 7	
			28 5	
	30	10	16	10
[illegible]	28 3	16 9	M_2 7 3[2]	7 3[1]

[1] Significantly different from the other values (0 01 < P < 0 05)
[2] M_2 significantly lower than M (P 0 01)

denervated cell population lacked measurable enzyme activity In cell bodies with measurable activity (both substrates) the values varied more than 10 fold from 2 7 to 31 1 × 10⁻⁴ $\mu l\ CO_2$/hr Five of 26 cells showed enzyme activity close to or exceeding 20 × 10⁻⁴ $\mu l\ CO_2$ hr Again two of the 10 cell bodies studied with MeCh as substrate showed distinctly higher AcChE activity than the remainder The ratio of AcCh MeCh hydrolysis was changed by denervation from 1 7 to 1 0

Discussion

Sympathetic ganglion cells of the cat similarly to those of other species (Giacobini 1957) differ widely in total ChE activity The majority have low but a few have rather high ChE activity Moreover the ganglion cells reveal the highest enzyme activity with acetylcholine AcCh as substrate This may be due to the fact that AcChE hydrolyzes AcCh more rapidly than acetyl β methylcholine (MeCh) In addition glial BuChE which hydrolyzes AcCh but not MeCh, may contribute to a small extent to the total ChE activity under both experimental conditions used BuChE occurs in capsule cells adjacent to the nerve cell bodies (Koelle 1951) and

histochemical studies suggest that its activity is unaffected by preganglionic denervation (Fredricsson and Sjoqvist 1962) It should be pointed out that variations in the amount of glia sticking to the cells can account only partly for the observed differences in ChE activity between individual ganglion cells In fact such differences were also demonstrated with acetyl β methylcholine as substrate, and it is known that AcChE does not occur in glial tissue of the cat's sympathetic ganglia (Koelle 1951)

Preganglionic denervation causes disappearance of AcChE from presynaptic terminals (Koelle 1951, Fredricsson and Sjoqvist 1962, Holmstedt, Lundgren and Sjoqvist 1963), but does not apparently affect the AcChE of the cell bodies (Fredricsson and Sjoqvist 1962, Holmstedt, Lundgren and Sjoqvist 1963) In this study the innervated ganglion cells had considerably higher ChE activity than the denervated cells Probably presynaptic terminals with high AcChE activity stick to the dissected innervated cell bodies

These facts explain why the Cartesian diver technique has revealed ChE activity in a relatively high proportion of *normal* sympathetic ganglion cells of several species (cf Giacobini 1956, 1957), a finding which has been quoted by Burn and Rand (1962) as indirect evidence for a cholinergic link in adrenergic transmission Our data reveal, however, that 80 % of the postganglionic cell bodies have low ChE activity and that in fact 35 % lack measurable activity If present at all, the enzyme activity in the latter cells can not exceed 10^{-5} μl CO_2/hr/cell equivalent to about 10^{-10} to 10^{-11} g AcCh/hr/cell This would correspond to about 1 μmole AcCh hydrolyzed/g nerve/hour, e g the lowest activity ever found in nerve tissue (cf Nachmansohn 1959)

When MeCh was used as substrate two out of 10 cells (both normal and denervated) showed markedly higher AcChE activity than the remainder (cf Table I) This figure would correspond roughly to the calculated proportion of cholinergic cell bodies in the 7th lumbar ganglion (cf Sjoqvist 1962 1963 b However, the possibility of a bimodal distribution of the AcChE values could not be tested statistically due to the limited number of sampled cells

The vast majority of the neurons in L7 appr 85 % are classified as adrenergic on the basis of their fluorescence for a monoamine probably noradrenaline Hamberger, Norberg and Sjoqvist 1965) This figure by far exceeds the proportion of neurons in which we failed to demonstrate AcChE around 35 % Our data thus confirm the view that a large proportion of adrenergic neurons contain AcChF (Koelle 1955 1966 Giacobini 1956 1957 Fredricsson and Sjöqvist 1962 Holmstedt Lundgren and Sjoqvist 1963) On the other hand they do not lend support to the concept that all sympathetic neurons contain this enzyme cf Burn and Rand 1962, Koelle 1962 Nachmansohn 1963)

It is important to appreciate species differences in the distribution of cholinesterases Sympathetic ganglion cells of the rat s superior cervical ganglion contain BuChE as well as AcChE Koelle 1951 Giacobini 1956 1957 In this species Franko 1966 recently demonstrated histochemical staining for a cholinesterase and fluorescence for monoamines in the same sympathetic ganglion cells A more or less un staining of most cell bodies is apparent from the photomicrographs but in con

ing preparations from the cat (Hamberger, Norberg and Sjoqvist 1965) heavily stained cell bodies are easily distinguished from the remainder (also cf Fig 1) There is at the moment no evidence for the existence of true cholinergic fibres, originating from cell bodies in the superior cervical ganglion of the rat Hamberger *et al* (1965), who examined the physiologically well-explored lumbo sacral ganglia of the cat, found no fluorescence for noradrenaline in those cell bodies with heavy staining for AcChE, which in all probability give rise to cholinergic sweat secretory fibres (Sjoqvist 1963 a, b) More recently Jakobowitz and Koelle (1965) demonstrated both catecholamine fluorescence and AcChE-activity in the same nerve trunk innervating the vas deferens of the guinea-pig However, in this study it could not be ascertained whether AcChE and catecholamine fluorescence were located within the same nerve fibre

In our experiments, denervation changed the ratio of AcCh/MeCh hydrolysis from 1 7 to 1 0 This may reflect a biochemical difference between the perikaryal and presynaptic enzyme, the latter being more efficient in hydrolyzing the physiological substrate acetylcholine

The present investigation supports the concept that sympathetic ganglia of the cat contain at least two functionally different cell populations, a smaller AcChE rich one (cholinergic) and a larger noradrenaline containing one (cf Sjoqvist 1962, 1963, Hamberger *et al* 1965) While a certain proportion of the latter lacks measurable AcChE activity, *a relatively large proportion contains this enzyme It remains* to be clarified whether *low* AcChE-activities in ganglionic cell bodies reflect the participation of acetylcholine in transmission in the corresponding nerve terminals This problem may possibly be further elucidated by parallel determinations of acetylcholine, cholinacetylase and AcChE in functionally different sympathetic ganglion cells

This work was supported by grants NB 04561 01 and 02 and G M 13970-01 from the U S Public Health Service by the Karolinska Institutet and the Swedish Medical Research Council project no 12X 246 01 and 02

References

Burn, J H and M J Rand A new interpretation of the adrenergic nerve fibres *Advanc Pharm Academic Press Inc (Lond)* 1962 *1* 1—30

Dale, H H and W Feldberg Chemical transmission of secretory impulses to sweat glands of cat *J Physiol* (Lond 1934 *82* 121—128

Eränkö O Demonstration of catecholamines and cholinesterases in the same section *Pharmacol Rev* 1966 *18* 353

Fredricsson B and F Sjoqvist A cytomorphological study of cholinesterase *in* sympathetic

[illegible] e cells *Acta physiol*

[illegible] sympathetic cells *J*

[illegible] and

[illegible] gic [illegible] rel

transmission ed by [illegible] Douglas and A [illegible]

Prague 1963 *3* 41—53 Pergamon Press Oxford (1965)

HOLMSTEDT B and F SJOQVIST, Distribution of acetocholinesterase in the ganglion cells of various sympathetic ganglia *Acta physiol scand* 1959 *47* 284—296

HOLMSTEDT, B, G LUNDGREN and F SJÖQVIST, Determination of acetylcholinesterase activity in normal and denervated sympathetic ganglia of the cat A biochemical and histochemical comparison *Acta physiol scand* 1963 *57* 235—247

JAKOBOWITZ D and G B KOELLE Histochemical correlations of acetylcholinesterase and catecholamine in the same nerve trunk *J Pharmacol exp Ther* 1965 *148* 225—237

KOELLE G B, The elimination of enzymatic diffusion artifacts in the histochemical localization of cholinesterases and a survey of their distribution *J Pharmacol exp Ther* 1951 *103* 153—1[illegible]1

KOELLE G B, The histochemical identification of acetylcholinesterase in cholinergic, adrenergic and sensory neurons *J Pharmacol exp Ther* 1955 *114* 167—184

KOELLE, G B, A new general concept of the neurohumoral functions of acetylcholine and acetylcholinesterase *J Pharmacol exp Ther* 1962 *14* 65—90

KOELLE, G B, Summary of discussion and commentary *Pharmacol Rer* 1966 *18* 359—365

NACHMANSOHN D, Chemical and molecular basis of nerve activity *Academic Press*, London 1959

Acta physiol. scand. 1967. 69. 362—372

From the Nobel Institute for Neurophysiology, Karolinska Institutet, Stockholm Sweden
and the Department of Physiology, University of Umeå, Sweden

Contractures of Single Slow Muscle Fibres of Xenopus Laevis Elicited by Potassium, Acetylcholine or Choline

By

JAN LÄNNERGREN

Received 12 November 1966

Abstract

LÄNNERGREN, J. *Contractures of single slow muscle fibres of Xenopus laevis elicited by potassium, acetylcholine or choline.* Acta physiol. scand. 1967. 69. 362—372.

Contractures were induced in single slow muscle fibres of *Xenopus laevis* by step increases in external potassium concentration or by application of solutions containing acetylcholine or choline. $[K]_o$ needed to evoke a contracture was lower in slow fibres (about 15 mM K) than in twitch fibres (about 25 mM-K). In $[K]_o$ near the mechanical threshold tension developed rapidly after a long initial delay. The relation between $[K]_o$ and peak tension was extremely steep. Evidence was [illegible]

whereas potassium depolarizes the cell membrane uniformely.

In addition to the common twitch or fast muscle fibres, some amphibian skeletal muscles contain so called slow fibres. Slow fibres differ from twitch fibres in several ways (Tasaki and Mizutani 1944, Kuffler and Vaughan Williams 1953 *a*, *b*, Burke and Ginsborg 1956, see also review by Peachey 1961): (a) they are supplied by a separate system of small diameter axons, (b) their cell membranes do not respond with a regenerative, all-or-none action potential to electrical stimulation but are only locally depolarized, (c) they give slow, graded contractions in response to stimulation of their supplying nerve fibres in contrast to the well-known twitch of fast fibres, (d) they respond with maintained contractures to the application of potassium rich solutions or solutions containing end plate depolarizing agents such as acetylcholine, instead of giving transient contractures or twitches as fast fibres do.

Important information about how the membrane potential controls production of tension in twitch muscle fibres was obtained by Hodgkin and Horowicz (1960) in a study of potassium contractures in single frog fibres. These authors found that

the development of tension was related to membrane potential by a steep S-shaped curve Similar findings have subsequently been obtained by Luttgau (1963), also in the frog and by Frankenhaeuser and Lannergren (1967) in the toad.

No corresponding investigation has been performed on isolated amphibian slow fibres Instead, most information about the mechanical properties of slow fibres has been derived from experiments on whole muscles or bundles of muscle fibres Since in Amphibia, no muscle exists that is composed of slow fibres only (Kuffler and Vaughan Williams 1953*b*) the analysis of the results is complicated by the possible contribution to tension production made by twitch fibres Luttgau (1963), in a few experiments with single slow fibres from the iliofibularis of the frog, studied the effect of changes in $[Ca]_o$ on contractures induced by one and the same high potassium solution

In the present investigation the mechanical responses of single slow fibres of the toad (*Xenopus laevis*) were studied in solutions with various concentrations of potassium or acetylcholine, particular attention being given to concentrations close to the mechanical threshold The effect of choline was also tested in some experiments

It will be shown that these slow fibres respond with long lasting contract [illegible]

[illegible]

[illegible] ological characterist of the muscle fibres in the iliofibularis muscle are such as to permit the selection slow fibres during dissection (Lannergren and Smith 1966)

Methods

Preparation The experiments were performed during October–March on *Xenopus laevis* of both sexes The animals were kept at room temperature and were fed [illegible] ed meat once a week The [illegible] muscle was excised and placed in Ringer's solution [illegible] nd held [illegible]

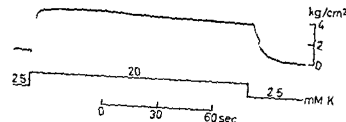


Fig 1 Long lasting contracture of a single slow fibre in 20 mM K 2 mM Ca Relaxation starts soon after change back to Ringer's solution Fibre 29 diameter 35 μm maximal tension 4 2 kg/cm²

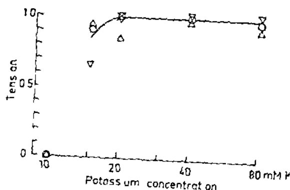


Fig 2 Relation between K-concentration (logarithmic scale) and peak tension Ordinates are given as fractions of amplitude of first contracture in 20 mM K (×) The other values at 20 mM K are controls during the experiment Interrupted part of curve is explained in the text △ fibre 1 diameter 50 μm maximal tension 5 1 kg cm² ▽, fibre 2 50 μm 3 1 kg cm ○, fibre 12 75 μm 4 8 kg cm²

constant by adding NaCl (b Solutions with acetylcholine 100 mg ACh Cl (F Hoffmann La Roche & Co A G Basel (stock solution)
with normal Ring
solution (without
stadt) on an equi

Class distilled water was used throughout The experiments were carried out at room tempera 20—24 C)

Results

Potassium contractures

When a solution of increased K concentration was applied the fibre contracted relatively rapidly and maintained tension as long as it remained in the high K solution (Fig 1) On return to Ringer's solution the fibre relaxed completely Up to twelve repeated contractures of 40—60 sec duration and nearly constant tension output could be obtained in one and the same fibre The maximum tension produced by 30 single slow fibres was 3 9 ± 0 8 kg/cm² (mean ± S D) This value is somewhat higher than the maximum tension of 3 1 ± 0 5 kg/cm² produced by 28 twitch fibres from the same muscle in Xen pus Frankenhaeuser and Lannergren 1967)

The relation between maximum tension development and external K-concentration was studied in a number of fibres Figure 2 shows the results of experiments on three fibres It can be seen that the slow fibres developed high tension even in moderate K concentrations i e 15 20 mM K and that in concentrations above 20 mM there was no further increase in tension development The general trend in the findings (Fig 2) agrees with corresponding results from twitch fibres (Hodgkin and Horowicz 1960, Frankenhaeuser and Lannergren 1967) The values for the slow

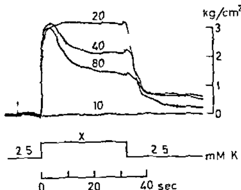


Fig 3 Photographically superimposed tension records taken from one fibre in various K-concentrations X denotes period of increased $[K]_o$, the concentration (mM) used is given above the tension lines The 10 mM K solution was left on somewhat longer than the three other solutions (note artifacts from fluid change) The records were taken in order 40, 10, 20 and 80 mM-K Fibre 3, diameter 60 μm, maximal tension 3 2 kg cm²

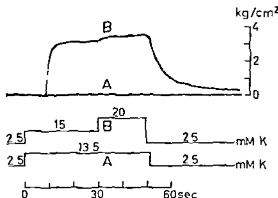


Fig 4 Contractures in $[K]_o$ near the mechanical threshold *A*, 13 5 mM K applied for 50 sec, no contracture *B*, 15 mM-K, rapid development of tension after an initial delay of 10 sec Small increase in tension at change to 20 mM K Fibre 17, diameter 50 μm, maximum tension 3 5 kg/cm²

fibres, however, lie more to the left in the graph relating peak tension to log $[K]_o$ and the maximum steepness of the slow fibre curve is clearly greater

The time-course of contractures in various potassium concentrations was also studied The rate of tension development was but little increased in 80 mM-K compared to 20 mM K When sufficiently high $[K]_o$ was applied for long periods the tension in the slow fibres decreased towards a plateau and this plateau was lower at higher K concentrations (Fig 3) In twitch fibres the rising phase of the contracture is more dependent on the K-concentration and the relaxation in corresponding solutions many times faster

Characteristics of the contracture near the mechanical threshold

It was found that no fibre contracted in 10 mM K and that all the fibres tested gave an almost maximal contracture in 15 mM-K The dependence of peak tension on potassium concentration was clearly so steep that it seemed worth-while to investigate in further detail the mechanical response of fibres in this concentration range The experiment shown in Fig 4 illustrates the steepness very clearly A solution with 13 5 mM-K caused no contraction (Fig 4 *A*), while a solution of 15 mM-K caused a contracture of nearly maximal amplitude (Fig 4 *B*) No concentration could 1 found which caused a contractu

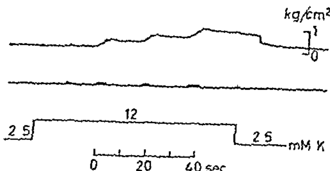


Fig 5 Tension resulting from local electrical stimulation (4 imp sec) of a fibre immersed in 12 mM K solution Upper line is tension broadening of lower line indicates periods of stimulation Fibre 22 diameter 75 µm, maximal tension 2 5 kg cm²

of the contracture in this concentration range was a very distinct delay that occurred before tension developed (Fig 4 see also Fig 6 The delay varied between 6 and 37 sec in experiments on six fibres It was remarkably constant in repeated tests with the same solution on one and the same fibre With increasing K-concentration the delay became shorter and negligible in 20 mM K

This behaviour — the large and sudden tension development after a long delay and the dependence of the delay on the strength of stimulation — is typical of a regenerative system, but can also occur without regeneration The fibre membrane might be sufficiently regenerative to cause this response although it is unable to propagate an all-or none action potential Alternatively, the regeneration might in some way involve the activity of the contractile system In both cases a full response ought to be triggered by local stimulation of a fibre which is depolarized to a level just below that required for a delayed response

Figure 5 illustrates an experiment designed to test this idea A solution with 12 mM K was applied and the fibre stimulated with short trains of current pulses at a frequency of 4 sec applied via platinum electrodes while the fibre was viewed in a dissecting microscope During stimulation the fibre could be seen to contract locally at the middle where the cathode was situated The resulting tension recorded at one tendon was small during the first short train of impulses When successive, longer trains were delivered the tension increased during each period of stimulation and attained a level which was held till the next period of stimulation When the solution with 12 mM K was replaced by Ringer s solution the fibre relaxed after a short delay The maximum tension obtained in this way ranged between 30 and 38 per cent of the tension developed in 20 mM K 3 fibres) It was never possible to start a contracture in the whole fibre by local stimulation provided the fibre was in good condition These findings appear inconsistent with a regenerative mechanism

It should be pointed out that a persistence of local contracture as seen in the microscope and reflected as a small maintained tension of the fibre, only occurred in a very narrow range of potassium concentrations, 2—3 mM below threshold In concentrations lower than 10 mM the stimulated region always relaxed after the cessation of stimulation When a fibre was stimulated locally in Ringer s solution

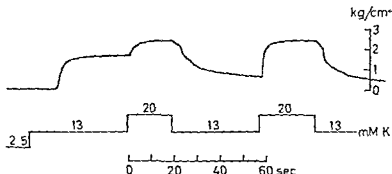


Fig 6 Tension produced by a fibre subjected to repeated changes in $[K]_o$. Note delay before first contracture appears and different tension levels attained in 13 mM K before and after the contracture in 20 mM K Fibre 22, diameter 75 μm maximal tension 2 5 kg cm²

(2 5 mM K) the resulting tension was exceedingly small The stiffness of the fibre was thus increased in the solutions with 10—12 mM-K In this connexion it may be noted that Frank (1965) found in twitch fibres different time courses for the development of stiffness and of tension

Fig 6 illustrates another type of experiment dealing with the same problem Changes were made between two solutions, 13 mM K and 20 mM K in this case, both of which caused a contracture when applied after Ringer s solution As can be seen in the Figure, the change from 13 to 20 mM K caused a moderate increase in tension When the solution surrounding the fibre was changed back to 13 mM K, however, the tension did not return to the previous level but declined progressively towards zero With a return to 20 mM K the fibre again produced maximum tension and then relaxed when 13 mM K was reapplied This inability of the fibre to maintain its tension in the lower potassium concentration after return from a higher one was also seen when changes were made from 15 to 20 to 15 mM K The maximum concentration in which relaxation took place after return from 20 mM K was not determined

These experiments were intended to reveal regeneration but failed to do so From the results of the last type of experiment it is also unlikely that the contractile system is largely saturated at concentrations below 20 mM K

Acetylcholine and choline contractures

The effect of applying Ringer s solution containing various amounts of ACh was tested in four fibres When concentrations of 10⁻⁶ g ml of ACh were used the fibre contracted rapidly and maintained a tension which was almost as large as that obtained in 20 mM K (Fig 7 B and C On return to normal Ringer's solution the fibre relaxed promptly In solutions of higher ACh-concentration (10⁻⁵ g ml) the maximum amplitude was the same but the tension declined slightly after 10 15 sec in resemblance to the behaviour observed in a 10 mM K solution In solutions of lower ACh-content the fibre contracted more slowly and the

Fig 7 *A* relation between ACh concentration (logarithmic scale) and peak tension Ordinates ... ACh except for fibre 25 where ... 70 μm maximal tension 3 9 kg ... kg/cm² △ fibre 27 ... μm 2 9 ... duced by 20 mM K (*B*) or ACh ... n in order *B* to *F* Control in 10 ...

Fig 8 *A* photographically superimposed records of contractures induced by choline The concentrations used are given in the Figure Measurements were taken in order 20 10 5 1 ... 1 75 mM choline R denotes Ringer's solution X period of choline application *B* control contracture in 20 mM K obtained after the contractures in *A* Fibre 21 diameter 70 μm maximal tension 3 9 kg cm²

of tension attained was lower Fig 7 *D*) The lowest ACh concentration required to cause a contracture was 5 × 10⁻⁸ to 10⁻⁷ g ml In contrast to the contractures resulting from the application of solutions with increased [K] just above threshold the contractures in low ACh-concentrations started immediately after the change of solution Also, when ACh was the stimulating agent there was no correspondence to the all-or none behaviour seen with potassium induced contractures, since the tension was smoothly graded with ACh concentration (Fig 7 *A*)

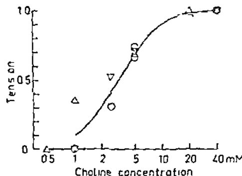


Fig 9 Relation between choline concentration (logarithmic scale) and peak tension Unit tension is height of contracture in 40 mM choline △ fibre 1, diameter 50 μm maximal tension 5 1 kg cm², ○, fibre 5 60 μm, 5 2 kg/cm², ▽, fibre 9 45 μm 3 6 kg/cm²

The ability of choline to induce contractures was also tested (six fibres) The idea to test this substance as well arose from experiments with a whole muscle, the ext dig longus III—IV (*Xenopus*) When Ringer's solution surrounding this muscle was changed to a solution containing choline Cl instead of NaCl the muscle contracted and maintained a tension of 5—10 % of the maximal It is known that this muscle in the frog contains slow fibres (*e g* Gray 1956, Lannergren 1965) and it seemed possible that these were responsible for the observed contracture

When a Ringer's solution with various amounts of sodium replaced by choline was applied to a slow fibre, a sustained contracture ensued with no tendency to decay during the time of application, 40—60 sec (Fig 8) When returned to normal Ringer's solution the fibre relaxed completely As with ACh the relaxation was slow when small concentrations were used Maximum tension was obtained in 10—20 mM choline Contractures in 20 and 40 mM had the same amplitude, but there was often a short delay in the onset of relaxation The maximum amplitude of the choline contractures was 50—95 % of that obtained in 20 mM-K The corresponding value for the ACh contractures was 94—100 % The minimum choline concentration sufficient to induce a contracture was found to be 1 0—2 5 mM (1 4—3 5 × 10^{-4} g/ml) Concentrations between 1 0 and 10 mM resulted in smoothly graded contractures with no delay in onset (Fig 8) The maximum tension is plotted against choline concentration in Fig 9

Discussion

The results presented here show that slow muscle fibres can respond with maintained contractures in solutions with elevated [K] or with ACh or choline This is consistent with earlier findings *e g* Pauschinger and Brecht 1961 Schaechtelin 1961, Lorkovic 1963, see also Kruger 1952) A discrepancy between the findings of Luttgau (1963) and those described here is apparent in the response to solutions of high [K] While the fibres studied by Luttgau maintained tension in a 100 mM K (hypertoni solution for 30 sec all the fibres investigated here relaxed considerably d a 30 sec application of a solution with 80 m

Luttgau It should be pointed out, however, that fibres were selected in different ways Those examined here were selected on the basis of their appearance in dark-field illumination, Luttgau selected fibres which remained contracted in a high-K solution

The potassium concentration required to initiate contractures was found to be about 15 mM-K The corresponding concentration for twitch fibres has been found to be higher, 20—30 mM-K (Hodgkin and Horowicz 1960), which is equivalent to a membrane potential of about — 50 mV Zacharova and Zachar (1965) found the same value of membrane potential for the mechanical threshold in the 'slow' fibres of the crayfish Kuffler and Vaughan Williams (1953) and Kiessling (1960) showed that slow fibres have a lower resting potential (about — 60 mV) than twitch fibres (— 94 mV, Hodgkin and Horowicz 1959) If it is assumed that activation occurs at the same absolute membrane potential in amphibian twitch and slow fibres, this would explain the difference in $[K]_o$ necessary to induce a contracture in the two types of fibre

The relation between tension and log $[K]_o$ found here for the slow fibres differs distinctly from that for the twitch fibres In the latter case the relation is described by an S-shaped curve with a smooth gradation of tension between 20 and 80 mM K (Hodgkin and Horowicz 1960, Frankenhaeuser and Lannergren 1967) For the slow fibres, present data indicate so steep a relation that it seems questionable whether tension is smoothly graded with membrane potential A discontinuity of the kind described might be explained on the basis of regeneration Experiments were made to disclose signs of regenerative activity but no such indications were found and one can only conclude that at the critical region a change of only a few millivolts in membrane potential was sufficient to change the state of the contractile system from quiescence to full activity This very steep characteristic seems to require that the membrane response is homogeneous along the whole fibre In support of this view it may be mentioned that one of the first signs of deterioration of the fibres was that their responses were more graded and in some of those cases visual observation revealed non-uniform contractions along the fibre

Let us further consider the possibility that membrane depolarization releases an activating substance in a continuous fashion and that a larger amount can be released than is required for maximal activation of the contractile system This would cause an increased steepness of the tension — membrane potential characteristic Since the fibres relaxed at the solution change from 20 mM K to 15 or 13 mM-K and since relaxation at the return to 2 5 mM K was largely unaffected by the preceding $[K]_o$ the experimental findings gave no indications in this direction

Thus the much steeper dependence of tension on $[K]_o$ in slow fibres compared to that in twitch fibres cannot be readily explained It should be pointed out, however, that the sarcotubular system thought to be involved in the inward spread of depolarization, has a different arrangement in slow fibres triads are very rarely found (Peachey and Huxley 1962 Page 1965 The matter is more fully discussed in a following paper Lannergren 1967

The contractures evoked by ACh in concentrations of 10^{-6} g/ml had almost the same amplitude and time-course as those in 20 mM K. A difference between the response to K and ACh was apparent, however, in concentrations just above threshold. While the K contractures began with a delay and rapidly reached a high level of tension the tension in ACh, also in low concentrations began immediately after the application of the test solution, the rate of rise increasing with the concentration. This behaviour was also seen when choline was used as the stimulating substance, the only differences being that larger concentrations were needed and that the contractures did not reach the same maximum amplitude.

The differences might be explained if it is assumed that in the ACh and choline *solutions the membrane is not homogeneously treated*, i.e. the membrane close to the nerve endings is more depolarized than the rest of the fibre resulting in contractures in only these parts. The tension development at local electrical stimulation of a fibre in 10—12 mM K indicates that potassium caused an increase in stiffness of the fibre at concentrations which did not cause a contraction (see Fig. 5.) It is likely that ACh and choline produced a similar increase in fibre stiffness. The graded nature of these contractures might then be explained on the basis of mechanical attenuation in a region of subthreshold depolarization. The full contracture seen with higher ACh concentration would be explained by successively larger parts of the fibre being depolarized past threshold level. That the nerve endings may not occupy the entire membrane area is indicated by observations of methylene blue preparations of *Xenopus* fibres (Lannergren and Smith 1966) and of frog fibres (Gray 1956).

This investigation was supported by grants from Karolinska Institutets Reservationsanslag, the Swedish Medical Research Council (Project Nos 14X—545—01 14X—545—02 and Stiftelsen Therese och Johan Anderssons Minne.

References

BURKE W and B L GINSBORG The electrical properties of the slow muscle fibre membrane *J Physiol* (Lond) 1956 *133* 586—598

FRANK G B Detection of two distinct processes in activation during potassium induced contractures of frog's skeletal muscle *Acta physiol scand* 1965 *65* 1—10

FRANKENHAEUSER B and J LANNERGREN The effect of calcium on the mechanical response of single twitch muscle fibres of *Xenopus laevis* *Acta physiol scand* 19[illegible] 69 24[illegible]

GRAY E G The spindle and extrafusal innervation of a frog muscle *Proc roy Soc B* 1956 *146* 416—430

HODGKIN A L and P HOROWICZ The influence of potassium and chl[illegible]ns on the membrane potential of single muscle fibres *J Physiol* (Lond) 1959 *148* [illegible]

HODGKIN A L and P HOROWICZ Potassium contractures [illegible] muscle fibres *J Physiol* (Lond [illegible] *153* 386 403

KIESSLING A Das Ruhepotential der tonischen Skeletmuskelfa[illegible]r des Frosches *Pflüg Arch ges Physiol* [illegible] *271* 124—138

KRUGER P *Tetanus und Tonus der quergestreiften Skelettmuskeln der Wirbeltiere und des Menschen* Akademische Verlagsgesellschaft Geest u Portig K G Leipzig 19[illegible]

LÄNNERGREN, J, Potassium contractures in single slow muscle fibres in *Xenopus laevis* *Acta physiol scand* 1966 *68* 277 127.

[illegible]
1966 *68* 263—274

LORKOVIC, H [illegible]
zeugenden [illegible]

LUTTGAU, H [illegible]
Physiol (Lo [illegible]

PAGE, S G, A comparison of the fine structures of frog slow and twitch muscle fibres *J cell Biol* 1965 *26* 477—497

PAUSCHINGER, P and K BRECHT, Influence of calcium on the potassium contracture of 'slow' and 'fast' skeletal muscle fibres of the frog *Nature* (Lond) 1961 *189* 583—584

PEACHEY, L D Structure and function of slow striated muscle In *Biophysics of physiological and pharmacological actions* Editor A M SHANES Amer Ass Advance Sci Washington 1961 pp 391—411

PEACHEY, L D and A F HUXLEY, Structural identification of twitch and slow striated muscle fibres of the frog *J cell Biol* 1962 *13* 177—180

SCHAECHTELIN, G, Der Einfluss von Calcium and Natrium auf die Kontraktur des M rectus abdominus *Pflug Arch ges Physiol* 1961 *273* 164—181

TASAKI, I and K MIZUTANI, Comparative studies on the activities of the muscle evoked by two kinds of motor nerve fibres *Jap J Med Sci Biophys* 1944 *10* 237—244

ZACHAROVA, O and J ZACHAR Contractions in single muscle fibres with graded electrogenesis *Physiol bohemoslov* 1965 *14* 401—411

Acta physiol. scand. 1967. 69. 373—382

From the Department of Physiology, University of Goteborg, Sweden

Fibre Components and Cortical Projections of the Elbow Joint Nerve in the Cat

By

H T. ANDERSEN,[1] L KORNER, S LANDGREN and H SILFVENIUS

Received 14 November 1966

Abstract

ANDERSEN, H T, L KÖRNER, S LANDGREN and H SILFVENIUS *Fibre components and cortical projections of the elbow joint nerve in the cat* Acta physiol scand 1967 *69* 373—382

A method is described for dissection and identification of the afferent elbow joint branch of the musculocutaneus nerve Conduction velocities of three components in the compound action

In studies of the somatosensory projections to the central nervous system it is important to consider the central responses to stimulation of muscle, joint and skin afferents Graded electrical stimulation of muscle and skin nerves has been frequently used in such studies and it is possible to stimulate the afferents from certain groups of receptors selectively by means of this method The usefulness of this feature was proven in studies of muscle afferents by Bradley and Eccles (1953 Laporte and Bessou (1957 and Eccles and Lundberg (1958 1959 The posterior knee joint nerve has been used as a sample of joint afferents in several investigations (*cf* Skoglund 1956) but joint afferents from the forelimb have so far only seldom been considered mainly because of the lack of a convenient forelimb joint nerve preparation

[1] Present address Institute of Nutrition Research, University of Oslo Norway

LÄNNERGREN, J, Potassium contractures in single slow muscle fibres in *Xenopus laevis* *Acta physiol scand* 1966 *68* 277 127

LÄNNERGREN, J, The effect of calcium on potassium contractures of single slow muscle fibres of *Xenopus laevis* *Acta physiol scand* 1967 *70* In press

LÄNNERGREN J and R S SMITH, Types of muscle fibres in toad skeletal muscle *Acta physiol scand* 1966 *68* 263—274

LORKOVIĆ, H, Akkommodation der tonischen Skeletmuskeln des Frosches auf die kontrakturerzeugenden Wirkungen von Kalium und Acetylcholin *Pflüg Arch ges Physiol* 1963 *278* 423—434

LUTTGAU, H C The action of calcium ions on potassium contractures of single muscle fibres *J Physiol* (Lond) 1963 *168* 679—697

PAGE, S G, A comparison of the fine structures of frog slow and twitch muscle fibres *J cell Biol* 1965 *26* 477—497

PAUSCHINGER, P and K BRECHT, Influence of calcium on the potassium contracture of 'slow and 'fast' skeletal muscle fibres of the frog *Nature* (Lond) 1961 *189* 583—584

PEACHEY, L D, Structure and function of slow striated muscle In *Biophysics of physiological and pharmacological actions* Editor A M SHANES Amer Ass Advance Sci Washington 1961 pp 391—411

PEACHEY, L D and A F HUXLEY, Structural identification of twitch and slow striated muscle fibres of the frog *J cell Biol* 1962 *13* 177—180

SCHAECHTELIN G, Der Einfluss von Calcium and Natrium auf die Kontraktur des M rectus abdominis *Pflüg Arch ges Physiol* 1961 *273* 164—181

TASAKI, I and K MIZUTANI, Comparative studies on the activities of the muscle evoked by two kinds of motor nerve fibres *Jap J Med Sci Biophys* 1944 *10* 237—244

ZACHAROVÁ O and J ZACHAR Contractions in single muscle fibres with graded electrogenesis *Physiol bohemoslov* 1965 *14* 401—411

brachialis Weak stimulation of the muscle branches always resulted in muscle twitches When Ejt was tested the stimulus strength could be raised to more than ten times this twitch threshold without visible muscular activity

The impulse discharge evoked in Ejt by joint mov[illegible] the humerus was held by two metal rods screwed [illegible] freely and could be flexed, extended and rotated [illegible] the action potentials were amplified, recorded or [illegible] speaker

In some of these experiments Ejt was dissected to the joint capsule which was opened The receptors were localized by light pressure with a glass rod applied outside the joint capsule as well as to its internal surface

[illegible] thresholds and conduction velocities the [illegible]

Results

The compound action potentials recorded from the musculocutaneus nerve in response to electrical stimulation of Ejt are shown in Fig 2 The maxima of three components are indicated by the arrows in the two monophasic records A and C Component 3 which was not seen in the triphasic record (B), was sometimes not separ[illegible] from the tail of component 2

The observed conduction velocities thresholds and maxima of the [illegible]

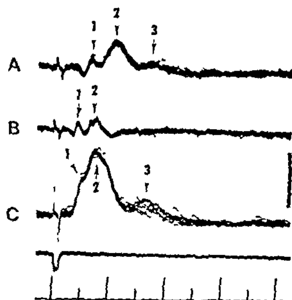


Fig 2 Records of action potentials recorded from n musculocutaneus in response to electrical stimulation of the elbow joint nerve A, monophasic record obtained proximally, B, triphasic and C, monophasic record obtained further distally on n musculocutaneus The distance between the two recording sites was 17 mm Figures and arrows indicate the three different components of the compound action potential The stimulating current pulse (11 μAmp 6 7 times threshold) is shown under record C Time scale 0 5 and 1 msec Voltage scale 0 1 m\

are given in Table 1 The threshold current necessary to evoke component 1 ranged between 1 2 and 2 0 (mean 1 5) μAmp Taking the threshold of component 1 as unit (T) the threshold of component 2 was 1 4 T and that of 3 was 2 3 T The variations may be seen in Table 1 Maximal amplitude of component 1 was reached at 1 8 T and of 2 at 2 9 T The maximum of 3 could not be accurately determined

The average conduction velocity of the fastest fibres in component 1 was 83 m per sec The corresponding values for 2 and 3 were 57 and 42 m per sec The slowest fibres responsible for the tail of the compound action potential had an average conduction velocity of 22 m per sec All three components were probably due to myelinated fibres As the duration of the stimulating pulse was only 100 μsec no C-fibre response was recorded in these experiments

TABLE 1 Conduction velocities and thresholds of the fibre groups in the elbow joint nerve

Date of exp	Conduction velocities (m sec of component 1	component 2	component 3	slowest fibres of response	Thresholds of (multiples of comp 1) comp 1	comp 2	comp 3	Stimulus strength of maximal response comp 1	comp 2
25 1 66	70	43		24	1 0	1 5			
27.1 66	87	65	49	20	1 0	1 4	1 9	2 5	4 1
8 3.66	67	47	38	19	1 0	1 5	1 7	1 2	1 9
10 3 66	85	71	52	18	1 0	1 3	1 9	1 4	2 0
22 3 66	100	64		35	1 0	1 4		2 3	3 4
25 3 66	91	54	27	18	1 0	1 5	3 5	1 6	3 0
Average	83	57	42	22	1 0	1 4	2 3	1 8	2 9

TABLE II Number of fibres and fibre diameters in the elbow joint nerve

	<3μ	3—4 9μ	5—6 9μ	7—9 9μ	10—14 9μ	15—19 9μ	Total
Exp 8 3 66	9	21	45	40	6	0	121
» 4 4 66	19	11	8	48	6	0	92
» 14 4 66	7	31	42	6	0	0	86
» 5 5 66	25	5	15	29	4	0	78
» 22 9 66	2	12	16	46	10	0	86
» 26 9 66	15	32	34	12	0	0	93
» 30 9 66	9	19	30	30	14	0	102
average nr of fibres	12 3	18 7	27 1	30 1	5 7	0	94
per cent	13	20	29	32	6	0	
Sasaoka (1939) per cent	25 58	16 28	54 01		4 13	0	
Nisimoto (1939) per cent	21	25	53		1	0	

The diameters of the Ejt fibres were measured on cross sections of the nerve from 7 expts The results are given in Table II The range of diameters observed was 1 3—13 5 μ The investigated Ejt nerves held 78—121 myelinated fibres The largest of them, with diameters between 10 and 13 5 μ, formed a small group including only 6 per cent of the fibres On the other hand fibres with diameters between 7 and 9 9 μ formed a substantial group comprising 32 per cent The origing of these two fibre groups will be discussed below The fibre diameter spectra given in the previous reports of Sasaoka (1939) and Nisimoto (1939) are shown for comparison at the bottom of Table II There is a fair agreement between the three sets of observations

The mass discharge recorded from the undivided Ejt nerve showed that the joint capsule receptors were slowly adapting mechanoreceptors sensitive to small flexions, extensions and rotations of the elbow joint The discharge frequency increased towards full extension and flexion of the elbow joint and had a minimum in an intermediate joint position The impulse frequency diagram of Fig 3 was obtained from a functional single fibre preparation with the receptor located in the capsule as shown by the response A to local pressure with a glass rod on the capsule Its response to a flexion of 5° shows a typical dynamic and a static response as seen in Fig 3 B

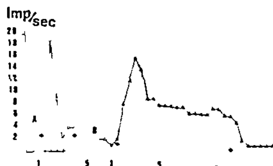


Fig 3 Diagram of the impulse frequency recorded from a functional single fibre preparation of the elbow joint nerve A Increase in frequency due to local pressure with a glass rod on the joint capsule (between arrows) B Response to flexion of the elbow joint from 90 to 85 at first arrow and back to 90 at second arrow

Cortical projections of Ejt

Electrical stimulation of Ejt evoked initially positive cortical surface potentials of short latency near the post cruciate dimple (Pcd) and in the forelimb areas of SI and SII As shown in Fig 4 these potentials were smaller in amplitude than those evoked by the skin afferents and by the Group I muscle afferents in the radial nerve This is to be expected in view of the rather small number of fibres in the Ejt nerve The Ejt potentials were evoked by single shock stimulation but improved in amplitude when a short train of shocks was used (cf Fig 4 A) The relatively largest Ejt responses were found near Pcd in the projection area of the Group I muscle afferents described by Oscarsson and Rosen (1963, 1966) and indicated by the broken line around point B in the cortical diagram of Fig 4 The area with initially positive Ejt potentials was often only 2—3 mm in diameter It is indicated in Fig 4 by the stippled field at arrow A, thus somewhat rostrally and laterally to the maximum of

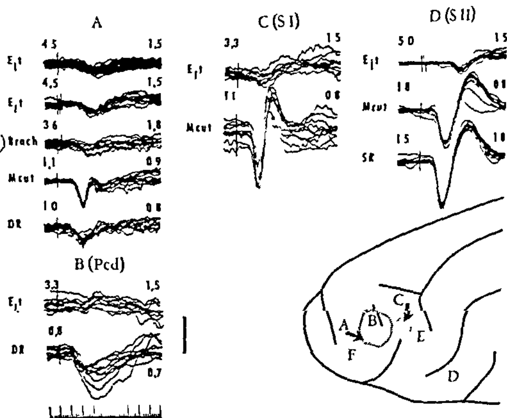


TABLE III Latencies and thresholds of initially positive cortical surface potentials evoked by electrical stimulation of the elbow joint nerve

Cortical area	Latency (msec)			Threshold[1]			Threshold[2]		
	Mean (SD)	Range	Nr of exp	Mean	Range	Nr of exp	Mean	Range	Nr of exp
Pcd region	8 5 (1 4)	7 0—11 0	8	2 0	1 0—4 5	7	0 7	0 5—0 9	6
Rostral SR	11 7 (2 5)	10 5—14 0	6	2 3	1 2—3 7	5	0 8	0 7—0 9	4
Caudal SR	9 5 (2 2)	6 5—12 0	8	2 4	0 8—4 1	7	0 9	0 5—1 5	5
SII forelimb area	11 2 (2 8)	7 5—12 5	6	3 2	1 7—4 3	5	1 1	0 6—2 0	4

[1] Threshold given in multiples of the stimulus strength necessary to evoke a threshold response in the musculocutaneus nerve
[2] Threshold given in multiples of the stimulus strength necessary to evoke maximal component 2 response in the musculocutaneus nerve

the Group I potential which was located in point B where the Ejt potential was mainly negative in polarity

The response to the skin afferents of the musculocutaneus nerve was a useful guide to the Ejt field The nerve branches to m brachialis on the other hand evoked rather small potentials in the Pcd region (cf records A in Fig 4)

The Ejt projection field in SI is indicated by the stippled area at arrow C in the diagram of Fig 4 A large initially positive potential was evoked here by the cutaneous branch of the musculocutaneus nerve (Fig 4 C, M cut) The Ejt field was, however, located somewhat rostrally to the centre of the caudal projection area of the superficial radial nerve (point E) Small initially positive Ejt potentials were found in the centre of the rostral projection area of the superficial radial nerve at point F These responses had a slightly longer latency This was generally also the case with the Ejt potentials evoked in the forelimb area of S II (Fig 4 D)

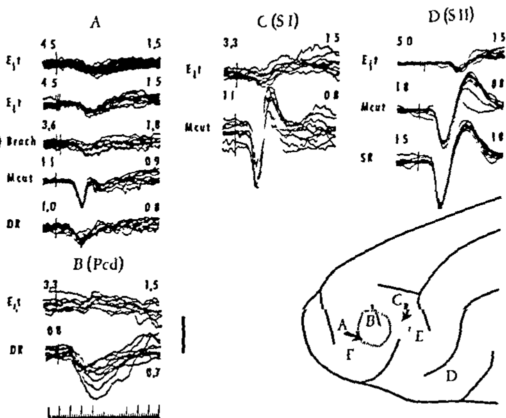
The latencies and the thresholds of the cortical Ejt responses are given in Table III The shortest latencies observed were around 7 msec They were found in responses recorded from the Pcd field from SI and occasionally, from SII The shortest observed Ejt latencies are therefore about 2 msec longer than those of the potentials evoked by the cutaneous afferents in SI and SII

As a rule the thresholds of the cortical Ejt potentials were higher than the threshold of component 1 in the musculocutaneus nerve but lower than the threshold of component 3 and lower than the stimulus strength necessary to evoke a maximal component 2 response in this nerve It was therefore concluded that the cortical responses were evoked by component 2 fibres *i e* by fibres with diameters between 7 and 10 μ and conduction velocities between 42 and 57 m per sec

In some of the preparations however, cortical potentials were evoked already by threshold stimulation of Ejt and in some animals only high threshold Ejt responses were observed

Cortical projections of Ejt

Electrical stimulation of Ejt evoked initially positive cortical surface potentials of short latency near the post cruciate dimple (Pcd) and in the forelimb areas of SI and SII As shown in Fig 4 these potentials were smaller in amplitude than those evoked by the skin afferents and by the Group I muscle afferents in the radial nerve This is to be expected in view of the rather small number of fibres in the Ejt nerve The Ejt potentials were evoked by single shock stimulation but improved in amplitude when a short train of shocks was used (cf Fig 4 A) The relatively largest Ejt responses were found near Pcd in the projection area of the Group I muscle afferents described by Oscarsson and Rosén (1963, 1966) and indicated by the broken line around point B in the cortical diagram of Fig 4 The area with initially positive Ejt potentials was often only 2—3 mm in diameter It is indicated in Fig 4 by the stippled field at arrow A, thus somewhat rostrally and laterally to the maximum of



in the large group of fibres from Ruffini endings in the joint capsule Nothing is known about the origin of our component 3 It is possible that the free nerve endings observed by Gardner (1944), Boyd (1954) and Skoglund (1956) could be the origin of these fibres

Gardner and Noer (1952), Gardner and Haddad (1953) and Skoglund (1956) have described cortical potentials evoked by electrical stimulation of the knee joint nerves Using natural stimulation Mountcastle (1957) has demonstrated that neurones in the SI area of the cat's cerebral cortex respond to movements of the limbs In a population of 685 neurones 105 were activated by such movements Although a part of this subgroup might have been discharged by Group I muscle afferents, it is obvious that some of them were activated from the joints as it was possible to localize the receptors to the joint capsule by dissection and local pressure Already from these results it was evident that joint afferents project to the cerebral cortex The question whether all the different groups of joint afferents contribute to the cortical projection path was, however, not solved Skoglund (1956) thus found evidence of low threshold knee joint afferent projections to the cortex, but Norrsell and Wolpow (1966) failed to confirm this observation They did not record any cortical potentials evoked by low threshold afferents in the posterior knee joint nerve, but found large responses to high threshold knee joint afferents

The question is of some significance as Skoglund (1956) suggests that the small group formed by the fastest joint afferents from the Golgi type endings is the one signalling joint position The more numerous fibres from the Ruffini endings were, on the other hand, assumed to signal joint movement The response of single afferent fibres from these endings was described by Skoglund (1956) and he found that they were sensitive to small changes in joint angle and that they covered a certain sector of joint movement with adapted discharge frequencies characteristic of each position within this sector The responsive sectors of the different fibres overlapped, and the number of active units increased with increasing extension and flexion being less numerous in a mid position Such receptors seem suitable for signalling both movement and position of the joint Their discharge was however, influenced by muscular contraction, and Skoglund therefore assumed that they could not be responsible for the perception of position but only for movement of the joint The effect of muscular contraction on the discharge from the Ruffini endings was however, not investigated quantitatively

The low threshold joint afferents from the Golgi and the Ruffini endings and their central projections have been considered to form the neural basis of kinesthesis (cf Mountcastle 1957, Rose and Mountcastle 1959) They would therefore be expected to have cortical projections and the disagreement about these projections was thus disturbing Our findings support the assumption that afferent fibres from Ruffini endings in the elbow joint capsule send projections to the cerebral cortex

the assumption that the numerous Ruffini afferents, in spite of their dependence on the state of muscular contraction, may be capable of signalling position as well as movement of the joint The other possibility would be to emphasize the positive finding of low threshold joint afferent projections observed in some of the animals and to assume that the lack of this evidence in other preparations was due to failure in finding the appropriate projection area This area could no doubt, be hidden in the cortical folds formed around the sulci Such hidden joint afferent projections were in fact observed in the anterior suprasylvian fold by Landgren Silfvenius and Wolsk (1967)

This work was supported by the Swedish Medical Research Council (Project No B67 14X 95 03)

References

ANDREW B L and E DODT The deployment of sensory nerve endings at the knee joint of the cat *Acta physiol scand* 1953 *28* 287—296

ANDREW B L The sensory innervation of the medial ligament of the knee joint *J Physiol* (Lond) 1954 *123* 241—250

BOYD I A and T D M ROBERTS Proprioceptive discharges from stretch receptors in the knee joint of the cat *J Physiol* (Lond) 1953 *122* 38—58

BOYD I A The histological structure of the receptors in the knee joint of the cat correlated with their physiological response *J Physiol* (Lond 1954 *124* 476—488

BRADLEY K and J C ECCLES Analysis of the fast afferent impulses from thigh muscles *J Physiol* (Lond) 1953 *122* 462—473

ECCLES R M and A LUNDBERG The synaptic linkage of direct inhibition *Acta physiol scand* 1958 *43* 204—215

ECCLES R M and A LUNDBERG Synaptic actions in motoneurones by afferents which may evoke the flexion reflex *Arch ital Biol* 1959 *97* 199—221

GARDNER E The distribution and termination of nerves in the knee joint of the cat *J comp Neurol* 1944 *80* 11 32

GARDNER E and R NOER Projection of afferent fibres from muscles and joints to the cerebral cortex of the cat *Amer J Physiol* 1952 *168* 437—441

GARD[illegible] ndleg
of th[illegible] 31—

HURSH[illegible]
139

LANDGREN S H SILFVENIUS and D WOLSK Somatic sensory paths to the second cortical projection area of the Group I muscle afferents *J Physiol* (Lond) 1967 In press

[illegible] I (fibre afferentes [illegible] *49* 1025—1037

[illegible] somatic sens[illegible]

[illegible] nfasern im Verlauf der periferen Nervenaste *Jap J med Sci I Anatomy* 1939 *7* 545—572

OSCARSSON O and I ROSEN Projection to cerebral cortex of large muscle-spindle afferents in [illegible] nerves of the cat *J Physiol* (Lond) 1963 *169* 924—945

[illegible] and

[illegible] t of the peripheral nervous [illegible] *of Physiology* Ed J Field

[illegible] lenkast *Jap J med Sci I*

SASAOKA S Uber das Kaliber der markhaltigen Nerven [illegible] *Anatomy* 1939 *7* 315 322

SKOGLUND S Anatomical and physiological studies of knee joint innervation in the cat *Acta physiol scand* 1956 *36* Suppl 124

Acta physiol scand 1967. 69. 383—384

From the Department of Anatomy, University of Helsinki, Finland

Localization of Noradrenaline and Acetylcholinesterase in the Taenia of the Guinea-pig Caecum

By

GUNNAR ÅBERG[1] and OLAVI ERÄNKÖ

Many studies have been published on physiological and pharmacological responses of the guinea pig taenia coli, often with controversial results (see Burnstock *et al* 1966) In the present study, histochemical methods were employed to approach the problem

Catecholamines were demonstrated using formaldehyde induced fluorescence (Eränkö 1955 Falck, 1962), in the present case noradrenaline is responsible Koelle s (1951) acetylthiocholine technique together with tetra isopropylpyrophosphoramide (iso OMPA) was employed for the demonstration of acetylcholinestrerase activity

Fig 1 shows fluorescence in a cross section of the caecum numerous adrenergic nerve fibres with intensely fluorescent synaptic varicosities are visible in the taenia (T), while fewer fibres are present in the circular muscle layer C In longitudinal sections of the taenia (Fig 4) the fluorescent fibres were seen to run parallelly with the smooth muscle fibres in close proximity to them These observations are in agreement with those made by Hollands and Vanov 1965)

An abundance of cholinesterase positive fibres was observed in the taenia (Fig 2) The smooth muscle cells of the taenia also showed some enzyme activity The cholinesterase positive fibres of the circular muscle were less frequent than those in the taenia Between the muscular layers the nerve trunks and the ganglion cells of the Auerbach plexus (P exhibited an intense acetylcholinesterase reaction cholinergic nerve fibres were observed to emerge from this plexus to the taenia and to the circular muscle

The Auerbach plexus was also intensely fluorescent Fig 1 This fluorescence was due to numerous fluorescent fibres with frequent synaptic varicosities in contact not only with non fluorescent ganglion cell group as observed earlier by Norberg (1964) and Jacobowitz 1965 but probably also with non fluorescent nerve fibres in the plexus Fig 3

Histochemical examination of ganglion free taenia strip preparations dissected with special care to include only the longitudinal taenia muscle as is customary for pharmacological experiments often revealed parts of the Auerbach plexus with ganglion cells surrounded by fluorescent bouton This observation makes understandable the variable results reported with drugs such as dimethylphenylpiperazinium see Burnstock *et al* 1966)

[1] Present address Department of Zoophysiology University of Göteborg Göteborg Swed

the assumption that the numerous Ruffini afferents, in spite of their dependence on the state of muscular contraction, may be capable of signalling position as well as movement of the joint The other possibility would be to emphasize the positive finding of low threshold joint afferent projections observed in some of the animals and to assume that the lack of this evidence in other preparations was due to failure in finding the appropriate projection area This area could, no doubt, be hidden in the cortical folds formed around the sulci Such hidden joint afferent projections were in fact observed in the anterior suprasylvian fold by Landgren, Silfvenius and Wolsk (1967)

This work was supported by the Swedish Medical Research Council (Project No B67 14X 95 03)

References

ANDREW B L and E DODT The deployment of sensory nerve endings at the knee joint of the cat *Acta physiol scand* 1953 *28* 287—296

ANDREW B L The sensory innervation of the medial ligament of the knee joint *J Physiol* (Lond) 1954 *123* 241—250

BOYD I A and T D M ROBERTS Proprioceptive discharges from stretch receptors in the knee joint of the cat *J Physiol* (Lond) 1953 *122* 38—58

BOYD I A The histological structure of the receptors in the knee joint of the cat correlated with their physiological response *J Physiol* (Lond) 1954 *124* 476—488

BRADLEY K and J C ECCLES Analysis of the fast afferent impulses from thigh muscles *J Physiol* (Lond) 1953 *122* 462—473

ECCLES R M and A LUNDBERG The synaptic linkage of direct inhibition *Acta physiol scand* 1958 *43* 204—215

ECCLES R M and A LUNDBERG Synaptic actions in motoneurones by afferents which may evoke the flexion reflex *Arch ital Biol* 1959 *97* 199—221

GARDNER E The distribution and termination of nerves in the knee joint of the cat *J comp Neurol* 1944 *80* 11—32

GARDNER E and R NOER Projection of afferent fibres from muscles and joints to the cerebral cortex of the cat *Amer J Physiol* 1952 *168* 437—441

GARDNER E and B HADDAD Pathways to the cerebral cortex for afferent fibres from the hindleg of the cat *Amer J Physiol* 1953 *172* 475—482

HURSH J B Conduction velocity and diameter of nerve fibres *Amer J Physiol* 1939 *127* 131—139

LANDGREN S [illegible]
area of the [illegible]

LAPORTE Y a[illegible]
d[illegible]

MA[illegible]

MO[illegible] matic sensory
cc[illegible]

NISIMOTO K Über die Kaliber und Zahlenschwankung der markhaltigen Nervenfasern im Verlauf der periferen Nervenaste *Jap J med Sci I Anatomy* 1939 *7* 343—372

OSCARSSON O and I ROSÉN Projection to cerebral cortex of large muscle spindle afferents in forelimb nerves of the cat *J Physiol* (Lond) 1963 *169* 924—945

OSCARSSON O and I ROSÉN Short latency projections to the cat's cerebral cortex from skin and muscle afferents in the contralateral forelimb *J Physiol* (Lond) 1966 *182* 164 181

NORRSELL U and E R WOLPOW An evoked potential study of different pathways from the hind limb [illegible]

in the large group of fibres from Ruffini endings in the joint capsule Nothing is known about the origin of our component 3 It is possible that the free nerve endings observed by Gardner (1944), Boyd (1954) and Skoglund (1956) could be the origin of these fibres

Gardner and Noer (1952), Gardner and Haddad (1953) and Skoglund 1956) have described cortical potentials evoked by electrical stimulation of the knee joint nerves Using natural stimulation Mountcastle (1957) has demonstrated that neurones in the SI area of the cat's cerebral cortex respond to movements of the limbs In a population of 685 neurones 105 were activated by such movements Although a part of this subgroup might have been discharged by Group I muscle afferents, it is obvious that some of them were activated from the joints as it was possible to localize the receptors to the joint capsule by dissection and local pressure Already from these results it was evident that joint afferents project to the cerebral cortex The question whether all the different groups of joint afferents contribute to the cortical projection path was, however, not solved Skoglund 1956) thus found evidence of low threshold knee joint afferent projections to the cortex, but Norsell and Wolpow (1966) failed to confirm this observation They did not record any cortical potentials evoked by low threshold afferents in the posterior knee joint nerve, but found large responses to high threshold knee joint afferents

The question is of some significance as Skoglund 1956 suggests that the small group formed by the fastest joint afferents from the Golgi type endings is the one signalling joint position The more numerous fibres from the Ruffini endings were on the other hand assumed to signal joint movement The response of single afferent fibres from these endings was described by Skoglund 1956 and he found that they were sensitive to small changes in joint angle and that they covered a certain sector of joint movement with adapted discharge frequences characteristic of each position within this sector The responsive sectors of the different fibres overlapped and the number of active units increased with increasing extension and flexion being less numerous in a mid position Such receptors seem suitable for signalling both movement and position of the joint Their discharge was however influenced by muscular contraction and Skoglund therefore assumed that they could not be responsible for the perception of position but only for movement of the joint The effect of muscular contraction on the discharge from the Ruffini endings was however not investigated quantitatively

The low threshold joint afferents from the Golgi and the Ruffini endings and their central projections have been [illegible] nsidered to form the neural basis of kinesthesis cf M[illegible] [illegible] R[illegible] and Mountcastle 1959 [illegible] would therefore be expected to have cortical pr[illegible] [illegible] the disagreem[illegible] about these projections was thus disturbing Our findings [illegible] the assumption that afferent fibres from Ruffini endings [illegible] the [illegible] capsule send projec[illegible] the cerebral cortex of the [illegible] [illegible] that the afferent from the Golgi endi[illegible] evoked cortical responses [illegible] of our animals From the point of [illegible] the hypothesis there seems to be two ways out of this dilemma One of these [illegible]

Discussion

The n musculocutaneus branch to the anterior part of the elbow joint capsule of the cat was described by Martin (1915) Its fibre diameter spectrum was studied by Sasaoka (1939) and Nisimoto (1939) We have confirmed the observations of these workers (*cf* Table II) Using the relationship between fibre diameter and conduction velocity described by Hursh (1939) the anatomical observations may be compared with the conduction velocities of the three components of the action potential in the elbow joint nerve Component 1 was due to joint afferents with a conduction velocity of about 60 to 80 m per sec This corresponds to fibre diameters of 10—13 μ The amplitude of this component was small which is in agreement with the observation of a small group of fibres between 10 and 13 5 μ The large component 2 with a conduction velocity between 40 and 60 m per sec agrees rather well with the large group of fibres with diameters between 7 and 10 μ The smaller component 3 with conduction velocities varying from 20 to 40 m per sec should consist of fibres with diameters between 3 and 7 μ and should correspond approximately to the 49 per cent of the fibres in the 3—4 9 and 5—6 9 μ groups These two groups thus include a comparatively large number of fibres but contribute a component of rather small amplitude to the compound action potential This is however in agreement with the assumption of a linear relationship between the fibre diameter and the amplitude of the action potential

The fibre diameter spectra of the posterior and medial knee joint nerves in the cat were studied by Gardner (1944) and Skoglund (1956) According to their observations the nerves contain a small group of fibres with diameters between 10 and 17 μ This group includes about 10 per cent of all fibres in the joint nerve Andrew (1954) and Skoglund (1956) identified the endings of these large fibres and found that they were Golgi endings located in the ligaments of the joint The impulse discharge evoked in the large afferents by joint movements was slowly adapting (Andrew and Dodt 1953 Skoglund 1956 The adequate stimuli were extension flexion or rotation of the knee joint but the response could be recorded also by stretch after sectioning of the ligament

Boyd and Roberts 1953) and Boyd (1954) found that many slowly adapting knee joint afferents originated in Ruffini endings of the type described by Gardner (1944) These endings were located in the joint capsule They were the most commonly observed ones and their fibre diameters were 7—10 μ Skoglund (1956) confirmed these observations and in his material the group 7—10 μ fibres included about 30 per cent of all the fibres in the knee joint nerves Fibres originating in modified Vater Pacini corpuscles also belonged to this diameter group but these receptors were rare

Assuming that the origin of the elbow joint nerve fibres are similar to those of the kneee joint it may be suggested that our component 1 of the compound action potential is due to activity in fibres originating in the Golgi endings of the joint ligaments In agreement with the observations of Gardner (1944) and Skoglund (1956) this component is small The large component 2 should be evoked by activity

in the large group of fibres from Ruffini endings in the joint capsule Nothing is known about the origin of our component 3 It is possible that the free nerve endings observed by Gardner (1944), Boyd (1954) and Skoglund (1956) could be the origin of these fibres

Gardner and Noer (1952), Gardner and Haddad (1953) and Skoglund (1956) have described cortical potentials evoked by electrical stimulation of the knee joint nerves Using natural stimulation Mountcastle (1957) has demonstrated that neurones in the SI area of the cat's cerebral cortex respond to movements of the limbs In a population of 685 neurones 105 were activated by such movements Although a part of this subgroup might have been discharged by Group I muscle afferents, it is obvious that some of them were activated from the joints as it was possible to localize the receptors to the joint capsule by dissection and local pressure Already from these results it was evident that joint afferents project to the cerebral cortex The question whether all the different groups of joint afferents contribute to the cortical projection path was, however, not solved Skoglund (1956) thus found evidence of low threshold knee joint afferent projections to the cortex, but Norrsell and Wolpow (1966) failed to confirm this observation They did not record any cortical potentials evoked by low threshold afferents in the posterior knee joint nerve, but found large responses to high threshold knee joint afferents

The question is of some significance as Skoglund (1956) suggests that the small group formed by the fastest joint afferents from the Golgi type endings is the one signalling joint position The more numerous fibres from the Ruffini endings were on the other hand, assumed to signal joint movement The response of single afferent fibres from these endings was described by Skoglund (1956 and he found that they were sensitive to small changes in joint angle and that they covered a certain sector of joint movement with adapted discharge frequences characteristic of each position within this sector The responsive sectors of the different fibres overlapped, and the number of active units increased with increasing extension and flexion being less numerous in a mid position Such receptors seem suitable for signalling both movement and position of the joint Their discharge was however, influenced by muscular contraction and Skoglund therefore assumed that they could not be responsible for the perception of position but only for movement of the joint The effect of muscular contraction on the discharge from the Ruffini endings was, however, not investigated quantitatively

The low threshold joint afferents from the Golgi and the Ruffini endings and their central projections have been considered to form the neural basis of kinesthesis (cf Mountcastle 1957 Rose and Mountcastle 1959) They would therefore be expected to have cortical projections and the disagreement about these projections was thus disturbing Our findings support the assumption that afferent fibres from Ruffini endings in the elbow joint capsule send projections to the cerebral cortex of the cat It is however still unsatisfactory that the afferents from the Golgi endings evoked cortical responses only in some of our animals From the point of view of the hypothesis there seems to be two ways out of this dilemma One of them involv

the assumption that the numerous Ruffini afferents, in spite of their dependence on the state of muscular contraction, may be capable of signalling position as well as movement of the joint The other possibility would be to emphasize the positive finding of low threshold joint afferent projections observed in some of the animals and to assume that the lack of this evidence in other preparations was due to failure in finding the appropriate projection area This area could, no doubt, be hidden in the cortical folds formed around the sulci Such hidden joint afferent projections were in fact observed in the anterior suprasylvian fold by Landgren, Silfvenius and Wolsk (1967)

This work was supported by the Swedish Medical Research Council (Project No B67 14X 95 03)

References

ANDREW B L and E DODT The deployment of sensory nerve endings at the knee joint of the cat *Acta physiol scand* 1953 *28* 287—296

ANDREW B L, The sensory innervation of the medial ligament of the knee joint *J Physiol* (Lond) 1954 *123* 241—250

BOYD I A and T D ... joint of the

BOYD I A ... their physic

BRADLEY K and J C ECCLES Analysis of the fast afferent impulses from thigh muscles *J Physiol* (Lond) 1953 *122* 462—473

ECCLES R M and A LUNDBERG The synaptic linkage of "direct inhibition *Acta physiol scand* 1958 *43* 204—215

ECCLES R M and A LUNDBERG Synaptic actions in motoneurones by afferents which may evoke the flexion reflex *Arch ital Biol* 1959 *97* 199—221

GARDNER E The distribution and termination of nerves in the knee joint of the cat *J comp Neurol* 1944 *80* 11—32

GARDNER E and R NOER Projection of afferent fibres from muscles and joints to the cerebral cortex of the cat *Amer J Physiol* 1952 *168* 437—441

GARDNER E and B HADDAD Pathways to the cerebral cortex for afferent fibres from the hindleg of the cat *Amer J Physiol* 1953 *172* 475—482

HURSH J B Conduction velocity and diameter of nerve fibres *Amer J Physiol* 1939 *127* 131—139

LANDGREN S H SILFVENIUS and D WOLSK, Somato-sensory paths to the second cortical projection area of the Group I muscle afferents *J Physiol* (Lond) 1967 In press

LAPORTE Y and P BESSOU Étude des sous groupes lent et rapide du groupe I (fibre afferentes d origine musculaire des grand diametre) chez le chat *J Physiol* (Paris 1957 *49* 1025—1037

MARTIN P *Lehrbuch der Anatomie der Haustiere* Stuttgart 1915 *2*

MOUNTCASTLE V B Modality and topographic properties of single neurons of cat s somatic sensory cortex *J Neurophysiol* 1957 *20* 408—434

NISIMOTO K Über die Kaliber und Zahlenschwankung der markhaltigen Nervenfasern im Verlauf der periferen Nervenaste *Jap J med Sci I Anatomy* 1939 *7* 343—372

OSCARSSON O and I ROSEN Projection to cerebral cortex of large muscle-spindle afferents in forelimb nerves f the cat *J Physiol* Lond 1963 *169* 924—945

OSCARSSON O and I ROSEN Shor ... and muscle afferents in the contralat ... d

NORRSELL L and E R WOLPOW ... limb to the somatosensory areas ... us

... lopment ... *of Physiology* Ed J Field

... elenkast *Jap J med Sci I Anatomy* 1939 ... 31 —322

SKOGLUND S Anatomical and physiological studies of knee joint innervation in the cat *Acta physiol scand* 1956 *36* Suppl 124

Acta physiol. scand. 1967. 69. 383—384

From the Department of Anatomy, University of Helsinki, Finland

Localization of Noradrenaline and Acetylcholinesterase in the Taenia of the Guinea-pig Caecum

By

GUNNAR ÅBERG[1] and OLAVI ERÄNKÖ

Many studies have been published on physiological and pharmacological responses of the guinea pig taenia coli, often with controversial results (see Burnstock *et al* 1966) In the present study, histochemical methods were employed to approach the problem

Catecholamines were demonstrated using formaldehyde induced fluorescence (Eranko 1955 Falck, 1962), in the present case noradrenaline is responsible Koelle's (1951) acetylthiocholine technique together with tetra isopropylpyrophosphoramide (iso OMPA) was employed for the demonstration of acetylcholinestrerase activity

Fig 1 shows fluorescence in a cross section of the caecum, numerous adrenergic nerve fibres with intensely fluorescent synaptic varicosities are visible in the taenia (T), while fewer fibres are present in the circular muscle layer (C) In longitudinal sections of the taenia (Fig 4), the fluorescent fibres were seen to run parallelly with the smooth muscle fibres, in close proximity to them These observations are in agreement with those made by Hollands and Vanov (1965)

An abundance of cholinesterase positive fibres was observed in the taenia (Fig 2) The smooth muscle cells of the taenia also showed some enzyme activity The cholinesterase positive fibres of the circular muscle were less frequent than those in the taenia Between the muscular layers, the nerve trunks and the ganglion cells of the Auerbach plexus (P) exhibited an intense acetylcholinesterase reaction cholinergic nerve fibres were observed to emerge from this plexus to the taenia and to the circular muscle

The Auerbach plexus was also intensely fluorescent (Fig 1) This fluorescence was due to numerous fluorescent fibres with frequent synaptic varicosities in contact not only with non fluorescent ganglion cell groups, as observed earlier by Norberg (1964) and Jacobowitz 1965 but probably also with non fluorescent nerve fibres in the plexus Fig 3

Histochemical examination of ganglion-free ' taenia strip preparations dissected with special care to include only the longitudinal taenia muscle, as is customary for pharmacological experiments often revealed parts of the Auerbach plexus with ganglion cells surrounded by fluorescent boutons This observation makes understandable the variable results reported with drugs such as dimethylphenylpiperazinium (see Burnstock *et al* 1966

[1] Present address Department of Zoophysiology University of Göteborg Göteborg Sweden

the assumption that the numerous Ruffini afferents, in spite of their dependence on the state of muscular contraction, may be capable of signalling position as well as movement of the joint The other possibility would be to emphasize the positive finding of low threshold joint afferent projections observed in some of the animals and to assume that the lack of this evidence in other preparations was due to failure in finding the appropriate projection area This area could, no doubt, be hidden in the cortical folds formed around the sulci Such hidden joint afferent projections were in fact observed in the anterior suprasylvian fold by Landgren, Silfvenius and Wolsk (1967)

This work was supported by the Swedish Medical Research Council (Project No B67 14X 95 03)

References

ANDREW B L and E DODT, The deployment of sensory nerve endings at the knee joint of the cat *Acta physiol scand* 1953 *28* 287—296

ANDREW, B L The sensory innervation of the medial ligament of the knee joint *J Physiol* (Lond) 1954 *123* 241—250

BOYD I A and T D M ROBERTS Proprioceptive discharges from stretch receptors in the knee joint of the cat *J Physiol* (Lond) 1953 *122* 38—58

BOYD I A The histological structure of the receptors in the knee joint of the cat correlated with their physiological response *J Physiol* (Lond) 1954 *124* 476—488

BRADLEY K and J C ECCLES Analysis of the fast afferent impulses from thigh muscles *J Physiol* (Lond) 1953 *122* 462—473

ECCLES R M and A LUNDBERG The synaptic linkage of direct inhibition *Acta physiol scand* 1958 *43* 204—215

ECCLES R M and A LUNDBERG Synaptic actions in motoneurones by afferents which may evoke the flexion reflex *Arch ital Biol* 1959 *97* 199—221

GARDNER E The distribution and termination of nerves in the knee joint of the cat *J comp Neurol* 1944 *80* 11—32

GARDNER E and R NOER Projection of afferent fibres from muscles and joints to the cerebral cortex of the cat *Amer J Physiol* 1952 *168* 437—441

GARDNER, E and B HADDAD Pathways to the cerebral cortex for afferent fibres from the hindleg of the cat *Amer J Physiol* 1953 *172* 475—482

HURSH J B Conduction velocity and diameter of nerve fibres *Amer J Physiol* 1939 *127* 131—139

LANDGREN S H SILFVENIUS and D WOLSK, Somato sensory paths to the second cortical projection area of the Group I muscle afferents *J Physiol* (Lond) 1967 In press

LAPORTE Y and P BESSOU Étude des sous groupes lent et rapide du groupe I (fibre afferentes d origine musculaire des grand diametre) chez le chat *J Physiol* (Paris 1957 *49* 1025—1037

MARTIN P *Lehrbuch der Anatomie der Haustiere* Stuttgart 1915 *2*

MOUNTCASTLE V B Modality and topographic properties of single neurons of cat s somatic sensory cortex *J Neurophysiol* 1957 *20* 408—434

NISIMOTO K Über die Kaliber und Zahlenschwankung der markhaltigen Nervenfasern im Verlauf der periferen Nervenaste *Jap J med Sci I Anatomy* 1939 *7* 343—372

OSCARSSON O and I ROSEN Projection to cerebral cortex of large muscle spindle afferents in forelimb nerves of the cat *J Physiol* (Lond) 1963 *169* 924—945

[illegible] cerebral cortex from skin and [illegible] the hind [illegible] al nervous [illegible] J Field [illegible] *med Sci I Anatomy* 1939 *7* 315—322

SKOGLUND S Anatomical and physiological studies of knee joint innervation in the cat *Acta physiol scand* 1956 *36* Suppl 124

Fig 1 Fluorescence due to noradrenaline in a cross section of the guinea pig caecum T taenia C circular muscle 50 Fig 2 Acetylcholinesterase activity in the guinea pig caecum P site of Auerbach s plexus 50 Fig 3 Detail of the Auerbach plexus 100 Fig 4 Longitudinal section of the taenia 100

Our observations indicate a rich innervation of the guinea pig taenia, firstly, by cholinergic fibres originating from the ganglion cells of the Auerbach plexus and, secondly, by postganglionic adrenergic fibres from sympathetic ganglia outside the gut wall which innervate (a) the ganglion cells (b) the cholinergic nerve fibres in the Auerbach plexus and (c) the smooth muscle of the taenia Since all these sites can be influenced by stimulation or drugs complicated effects can be obtained

Work supported by research grants from the Finland State Commission of Medical Sciences the Sigrid Juselius Foundation and AB Bofors Molndal Sweden

References

BURNSTOCK G G CAMPBELL and M J RAND The inhibitory innervation of the taenia of the guinea pig caecum *J Physiol Lond* 1966 *182* 504—526
ERÄNKÖ O Distribution of fluorescing islets adrenaline and noradrenaline in the adrenal medulla of the hamster *Acta endocrin Kbh* 1955 *18* 174—179
[illegible] cellular localization of monoamines by a fluores- [illegible] ppl 197
[illegible] of catechol amines in visceral organs and ganglia [illegible] *macol* 19[illegible] *25* 307—316
JACOBOWITZ D [illegible] autonomic innervation of the gut *J Pharmacol exp* [illegible]
[illegible] of ch lines [illegible] 153—171